

University of Windsor

## Scholarship at UWindor

---

Electronic Theses and Dissertations

Theses, Dissertations, and Major Papers

---

1-1-1979

### Bead reagents for membrane proteins.

Linda Mei Chien Chen  
*University of Windsor*

Follow this and additional works at: <https://scholar.uwindsor.ca/etd>

---

#### Recommended Citation

Chen, Linda Mei Chien, "Bead reagents for membrane proteins." (1979). *Electronic Theses and Dissertations*. 6722.  
<https://scholar.uwindsor.ca/etd/6722>

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email ([scholarship@uwindsor.ca](mailto:scholarship@uwindsor.ca)) or by telephone at 519-253-3000ext. 3208.

# Bead Reagents for Membrane Proteins

By

LINDA MEI CHIEN CHEN

A Thesis

Submitted to the Faculty of Graduate Studies through the  
Department of Chemistry in Partial Fulfillment  
of the Requirements for the Degree of  
Master of Science at the  
University of Windsor

Windsor, Ontario, Canada

1979

UMI Number: EC54710

## INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI<sup>®</sup>

---

UMI Microform EC54710

Copyright 2010 by ProQuest LLC

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

---

ProQuest LLC  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106-1346

HR 3276

© LINDA MEI CHIEN CHEN 1979

727763

## ABSTRACT

The synthesis of long-arm thiolated beads from beaded polyacrylamide ( $\leq 400$  mesh,  $10\text{--}37\mu\text{M}$  diameter, 2,000 Dalton inclusion limit) is described. The long spacer arm, polyethylene glycol di(3-mercapto-propionate)(PEG-DMP) was introduced via thioether formation with haloacetamidoethyl beads. The latter were prepared via the N-hydroxysuccinimide ester (NHS) after surveying a number of peptide coupling methods. Iodide was chosen as the leaving group following a survey of the corresponding chloro-, bromo- and iodo-bead derivatives. The thiol group densities in the range  $3\text{--}7\mu\text{eq/g}$  of dry beads were achieved in the final derivative as determined by spectrophotometric titration. Radiochemical titration with  $[1\text{--}^{14}\text{C}]\text{-iodoacetamide}$  indicated sulfhydryl group density about 40% of the spectrophotometric one. Thiol beads were characterized as the mixed disulfides with thio-nitrobenzoic acid and 2-thiopyridone.

Characterization of long-arm thiolated beads in thiol-disulfide interchange reactions was made by the use of bead-S-S-2-Py coupling with 2-mercaptoethanol, PEG-DMP, papain, BSA-SH and cell (ghost). In papain experiments, with bead-S-S-2-Py in large excess (40-50-fold) over protein thiol, thiopyridone release corresponding to  $\geq 70\%$  of protein thiol groups occurred rapidly and this exceeded amounts of

coupled protein released by reductive cleavage ( $\leq 30$  nmol/g beads) by 3-10-fold. In analogous Bovine serum albumin (BSA) experiments, protein in the cleavage supernatant was 20- to 55-fold lower than expected from thiopyridone release. Variation of pH and/or coupling times did not alter the situation. However, when the mode of coupling was reversed, bead-SH coupling with BSA-S-S-2-Py, amounts of protein in the Dithioerythritol (DTE)cleavage supernatant were greater than 30 nmol per gram of dry beads. In erythrocyte ghost coupling with bead-S-S-2-Py, the amount of protein found in the cleavage supernatant was 0.40 mg/g beads which was 6.1% of the amount of protein in this experiment.

**DEDICATION**

**TO**

**MY PARENTS & HUSBAND**

## ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. K.E. Taylor, for his patient direction and encouragement during the course of this work.

I would also like to thank Y.C. Wu for her synthesized compound and J. Artiss for his help and advice.

I am indebted to my husband, K.S. Chen, for his supporting, understanding and preparing of the schemes and Mr. C.S. Yang for the typing of this work.



## TABLE CONTENTS

	PAGE
ABSTRACT .....	ii
DEDICATION .....	iv
ACKNOWLEDGEMENTS .....	v
LIST OF SCHEMES .....	xi
LIST OF TABLES .....	xiii
LIST OF APPENDICES .....	xiv
LIST OF ABBREVIATIONS .....	xv
 CHAPTER I INTRODUCTION .....	 1
 CHAPTER II EXPERIMENTAL .....	 13
A. Materials .....	13
B. Equipment .....	14
C. Methods .....	15
1. Analytical Methods .....	15
a. TNBS Test .....	15
b. Dry Weight Determination .....	15
c. Determination of Aliphatic Amino Groups .....	 16
d. DTNB Titration of Beads .....	16
e. Conversion of Beads to Bead-S-S-2-Py Form and 2-Py-S-S-2-Py titration of Beads .....	  17

	PAGE
2. Alkylating Study .....	18
3. Synthesis of the Long Arm Thiolated Polyacrylamide Beads .....	18
a. Preparation of Aminoethyl Polyacrylamide Beads .....	18
b. Preparation of Iodoacetamidoethyl- polyacrylamide from Aminoethylated Polyacrylamide Beads .....	19
c. Blocking Unreacted Amino Group of Iodoacetamidoethylpolyacrylamide Beads .....	19
d. Preparation of Long Arm Thiolated Beads from Iodoacetamidoethylpoly- acrylamide Beads .....	20
4. [1- <sup>14</sup> C]-Iodoacetamide Coupling with Bead-SH .....	21
5. Papain Coupling with Bead-S-S-2-Py .....	22
a. Preparation of Activated Papain .....	22
b. Activated Papain Coupling with Bead-S-S-2-Py .....	22

	PAGE
6. BSA Coupling with Bead-SH .....	23
a. BSA Coupling with Bead-S-S-2-Py .....	23
b. BSA-S-S-2-Py Coupling with Bead-SH ..	24
7. Cell Coupling with Bead-S-S-2-Py .....	25
a. Ghosts Reaction with 2-Acetamido-4- mercaptobutyric Acid Hydrazide (Thiol Hydrazide) .....	25
b. Ghost-Hydrazone Coupling with Bead-S-S-2-Py .....	26
8. SDS Polyacrylamide Gel .....	27
CHAPTER III RESULTS .....	28
A. Preparation of Long-Arm Thiolated Polyacrylamide Beads .....	28
1. Alkylation Study .....	28
2. Aminoethylpolyacrylamide Beads .....	29
3. Haloacetamidoethylpolyacrylamide Beads ..	29
4. Alkylation of $\alpha,\omega$ -dithiols using Haloacetamidoethyl Beads .....	35
5. Summary of Overall Synthesis of Long- Arm Thiolated Beads .....	40

B. Characterization of Long-Arm Thiolated Beads in Thiol-Disulfide Interchange Reactions .....	40
1. Activated Thiolated Beads, Bead-S-S-Ar .	44
2. Accessibility of Bead-S-S-2-Py to Small Thiols .....	44
3. [1- <sup>14</sup> C]-Iodoacetamide Alkylation of Bead-SH .....	45
4. Coupling of Papain to Bead-S-S-2-Py ....	48
5. Coupling of BSA-SH to Bead-S-S-2-Py ....	51
6. BSA-SH of High Thiol Titre and BSA-S-S-2-Py .....	55
7. BSA-S-S-2-Py Coupling with Bead-SH .....	56
8. BSA-S-S-2-Py Reaction with BSA-SH .....	58
9. Cell Coupling .....	58
CHAPTER IV DISCUSSION .....	61
A. Preparation of Long-Arm Thiolated Beads ...	61
B. Thiol-Disulfide Interchange and Alkylation Reactions of Long-Arm Thiolated Beads .....	65

	PAGE
APPENDIX 1 .....	71
APPENDIX 2 .....	72
APPENDIX 3 .....	74
REFERENCES .....	75
VITA AUCTORIS .....	80

## LIST OF SCHEMES

SCHEME	PAGE
I. Functionalization of Aminoalkyl Agarose	3
II. Thiol-Disulfide Interchange Reactions Involved in Covalent Chromatography	5
III. Application of the 2-thiopyridyl Group to Covalent Chromatography	7
IV. Protein-Protein Conjugation Using N-succinimidyl 3-(2-pyridyldithio) Propionate	9
V. Plan for Construction of Long-Arm Thiolated Polyacrylamide Beads	30
VI. Chloroacetylation of Aminoethyl Polyacrylamide Beads Using 1-ethyl-3(3-dimethyl-amino-propyl)- carbodiimide (EDAC)	33
VII. Chloroacetylation of Aminoethyl Polyacrylamide Beads Using Dicyclohexylcarbodiimide (DCC) and N-hydroxy-succinimide (NHS)	34
VIII. Chloroacetylation of Aminoethyl Polyacrylamide Beads Using N-ethyl-5-phenyl-isoxazolium-3'- sulfonate (Reagent K)	36

SCHEME	PAGE
IX.      Reaction of Haloacetamidoethylpolyacrylamide with dithiols	38
X.       Pathway for the Synthesis of Long Arm Thiol Beads from Polyacrylamide Beads	42
XI.      Reaction of Ghost with Thiol Hydrazide	59

## LIST OF TABLES

TABLE	PAGE
I. Alkylation Study with Polyethylene Glycol Di(3-mercaptopropionate)	31
II. Coupling Aminoethyl Polyacrylamide Beads and Haloacetic Acid	37
III. Reaction of Haloacetamidoethylpolyacrylamide with Polyethylene Glycol Di(3-mercaptopropionate)	39
IV. Comparison of Short Arm and Long Arm Thiolated Beads	41
V. Synthesis of Long Arm Thiolated Beads from Polyacrylamide Beads	43
VI. 2-mercaptoethanol Coupling with Bead-S-S-2-Py	46
VII. [ $^{14}\text{C}$ ]-Iodoacetamide Coupling with Bead-SH	47
VIII. Coupling of Papain with Bead-S-S-2-Py	49
IX. Coupling of BSA-SH with Bead-S-S-2-Py	52
X. Coupling of BSA-SH with Bead-S-S-2-Py	54
XI. BSA-S-S-2-Py Coupling with Bead-SH	57



## LIST OF APPENDICES

APPENDIX	PAGE
1. Quench Correction Curve Using A Set of $^{14}\text{C}$ Quenched Standards from Nuclear Chicago	71
2. (a) Calculation of Functional Group Density on Bead Surface	72
(b) Calculation of Packing of Spherical Protein Molecules on Surfaces of Spherical Beads	73
3. BSA Standard Curve Using Coomassie Brilliant Blue G-250 of Bradford's Method	74

## LIST OF ABBREVIATIONS

A	Absorbance
Ac <sub>2</sub> O	Acetic Anhydride
BSA	Bovine Serum Albumin
DCC	Dicyclohexylcarbodiimide
DTE	Dithioerythritol
DTNB	5,5'-Dithio-bis-(2-nitrobenzoic acid)
EDAC	1-Ethyl-3(3-dimethyl-amino-propyl)- carbodiimide
EDTA	Ethylenediamine Tetraacetate
Et <sub>3</sub> N	Triethylamine
NHS	N-Hydroxysuccinimide
PBS	Phosphate Buffer Saline
PEG-DMP	Polyethylene Glycol Di(3-mercapto- propionate)
Py-2-SH	2-Thiopyridone
Reagent K	N-Ethyl-5-phenyl-isoxazolium-3'- sulfonate
SDS	Sodium Dodecyl Sulfate; Lauryl Sulfate
Thiol Hydrazide	2-Acetamino-4-mercaptobutyric Acid Hydrazide
{1- <sup>14</sup> C}-Iodoacetamide	{ <sup>14</sup> C}-Iodoacetamide
2-Py-S-S-2-Py	Aldrithiol-2; 2,2-Dithiobis(pyridine); 2,2'-Dipyridyl Disulfide

## CHAPTER I

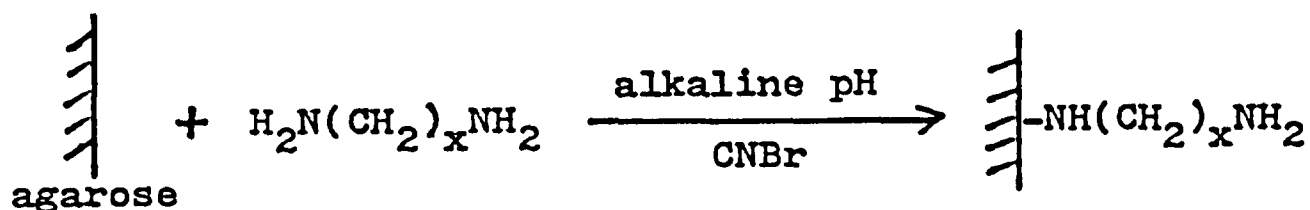
### INTRODUCTION

Campbell and his colleagues (1) in 1951 described a method related in principle to the use of "immunoabsorbents" introduced as chemically defined materials for chromatographic separation of antibodies. In the following year many authors applied these principles in the purification of nucleotides (2), certain species of transfer RNA (3) and enzymes (4,5) by utilization of hydrophobic polystyrene or hydrophilic cellulose polymers for the insoluble support matrix. Until 1969 only limited success (6,7,8) was achieved in the application of these techniques to the purification of enzymes (4,5), perhaps because the ligand was bound directly to the matrix and macromolecules could not approach the ligand. This problem of steric hindrance was resolved by placing a "spacer arm" between the matrix and ligand (9).

It has been shown that affinity chromatography is based on specific interactions between matrix-bound ligands and soluble biochemicals. In principle, a specific ligand interacting with a particular substance, usually a macromolecule, is covalently bound to an insoluble support. Substances with no affinity for the ligand will pass unretarded through a column of the bound support, otherwise the interacting materials will be retarded. A specific and

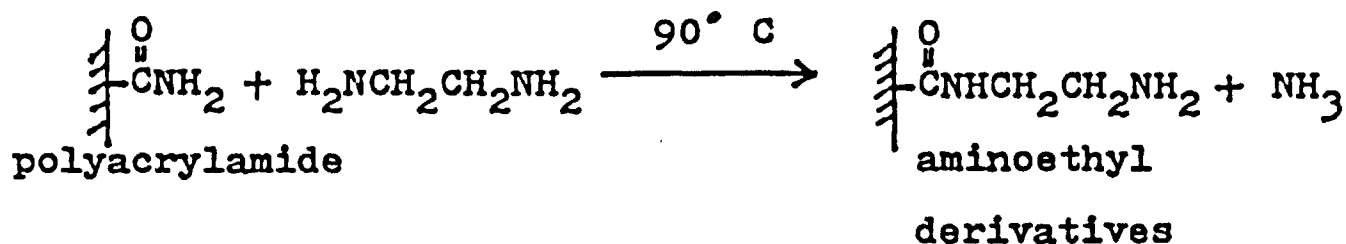
selective absorbent (matrix beads) must be deliberately prepared for every enzyme (10,11). Recently, the application of affinity chromatography for isolation, purification and separation of macromolecules, such as enzymes (12), proteins (13), antibodies (14) and DNA (15), has become a widely used tool.

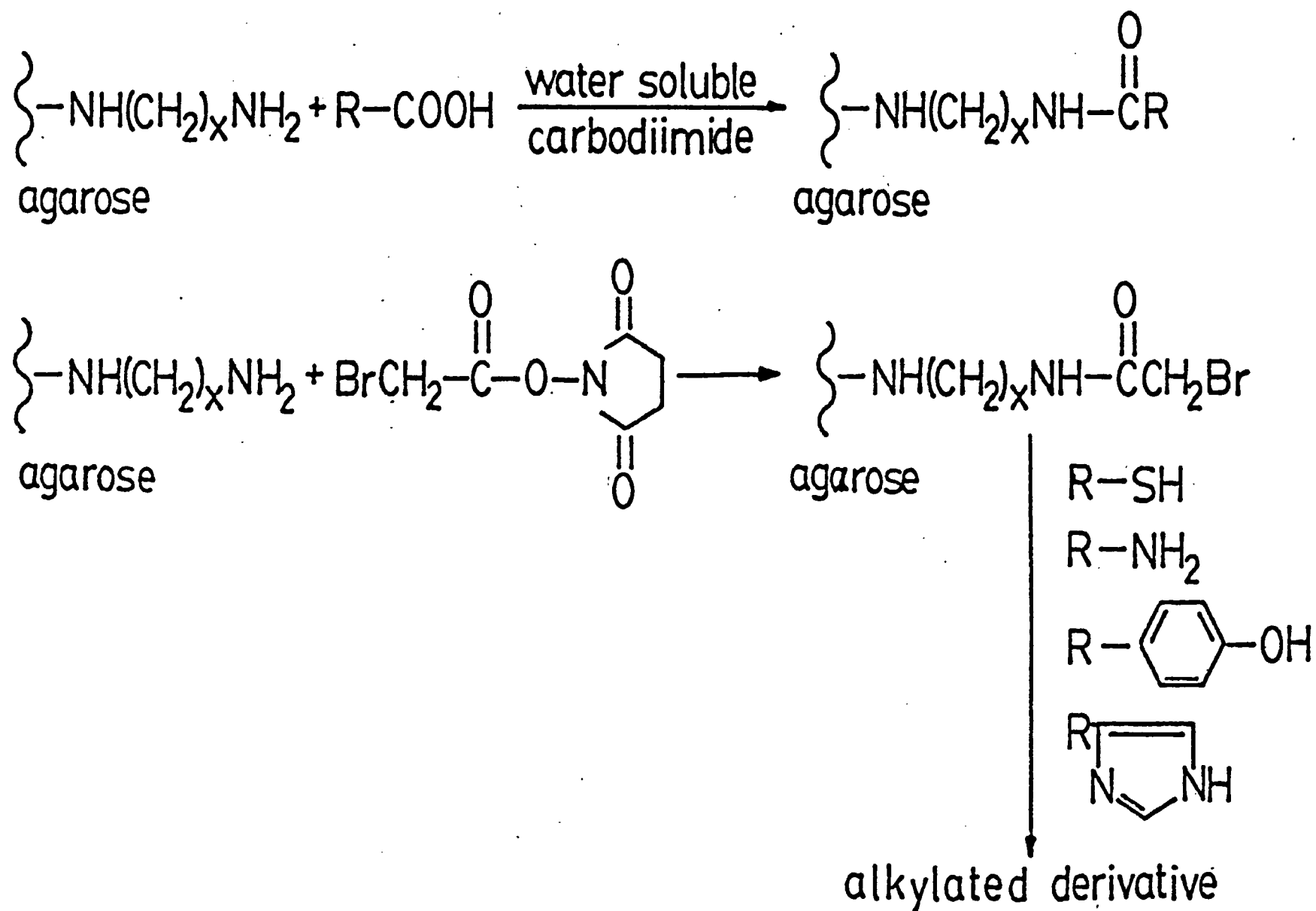
Beaded agarose is the most frequently used matrix because of its relative stability, biological inertness, superior chromatographic properties and facile activation with cyanogen bromide (9).



The most versatile way of constructing the "arm" is by  $\omega$ -aminoalkylation of agarose (16), followed by reactions such as Scheme I.

Inman and Dintzis (17) showed a number of useful chemical derivatizations of cross-linked polyacrylamide beads.



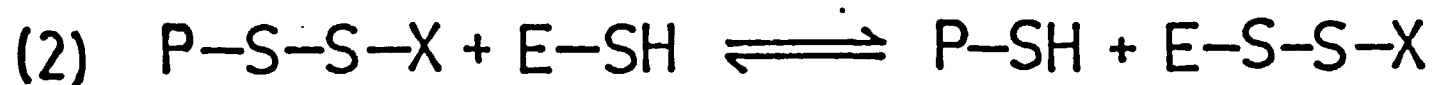


Scheme I : Functionalization of Aminoalkyl agarose

The coupling reactions used for aminoalkyl-agarose derivatives have been applied to acrylamide beads (18). They also introduced the use of reactive bead derivatives for the covalent binding of proteins as exemplified by the coupling of trypsin and of bovine serum albumin to beads.

A rather special type of affinity chromatography can be envisaged in which the material of the chromatography column reacts chemically with only one of the components of a mixture. After removal of the other components from the column by washing, the covalently bound component is released by reaction with a suitable reagent. The reaction that releases the component of interest should preferably leave the column in a form that can be readily "reactivated". This type of chromatography, which relies upon the scission and formation of covalent bonds, has been called "covalent chromatography" (19).

One particular type of covalent chromatography is based on thiol-disulfide interchange. For example, a thiol-containing protein (ESH) reacts with a polymer containing mixed disulfide residues (P-S-S-X) by thiol-disulfide interchange (Scheme II). For the purpose of isolation of ESH from a mixture, P-S-S-X must be chosen such that (i) the reaction of Scheme II(1) predominates over that of Scheme II(2); (ii) the reaction of Scheme II(1) is effectively irreversible; (iii) release of the thiol, XSH, is readily detected, for

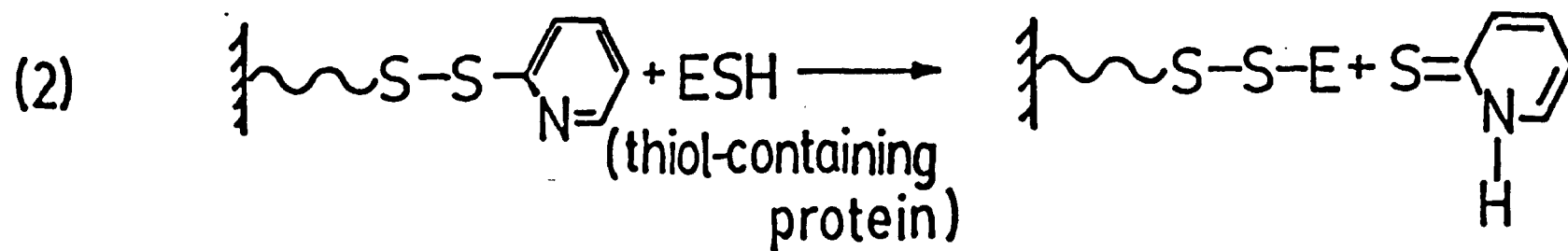
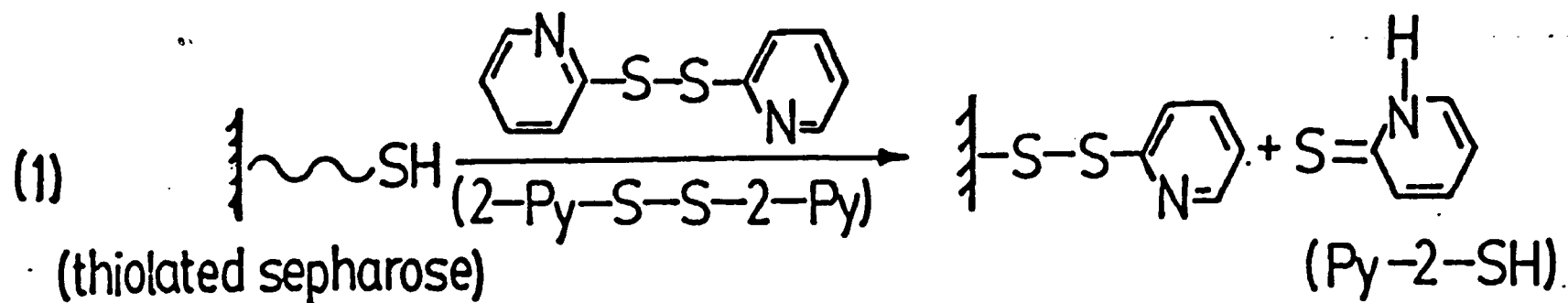


Scheme II: Thiol-disulfide Interchange Reactions Involved  
in Covalent Chromatography.

example, by spectrophotometry. Thus the attachment of a thiol-containing protein to the column could be followed by monitoring the release of XSH produced in the reaction of Scheme II(1). Subsequent elution with a solution of a low molecular weight thiol (RSH) would be expected to produce protein containing 1 mole of reactive thiol per mole of protein, the thiolated column (PSH) and the disulfide derivative of the low molecular weight thiol (R-S-S-R) (Scheme II(4))(20).

Brocklehurst and Little (21) found 2,2'-dipyridyl disulfide (2-Py-S-S-2-Py) to be a convenient and accurate active-site titrant for papain at pH 3.8 even in the presence of up to 10-fold molar excess of L-cysteine and up to 100-fold molar excess of 2-mercaptoethanol. And they also found that 2-Py-S-S-2-Py reacts with both low molecular thiols and papain to provide release of Py-2-SH, stoichiometric with the thiol content of the reactants. This suggested that reaction of the thiol-containing protein (ESH) with a polymer containing mixed disulfide residue (P-S-S-X) might be similarly effectively irreversible. Thus Brocklehurst et al. (20) chose Py-2-SH as XSH for the preparation of any thiol-containing protein (Scheme III). This method has been used for the preparation of fully active papain from dried papaya latex and separation of papain from L-cysteine (19, 20).





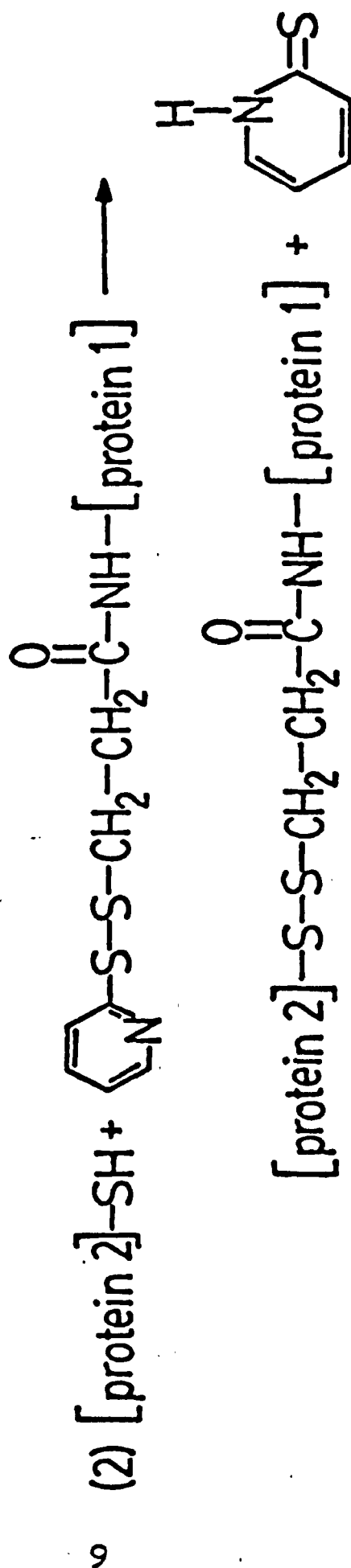
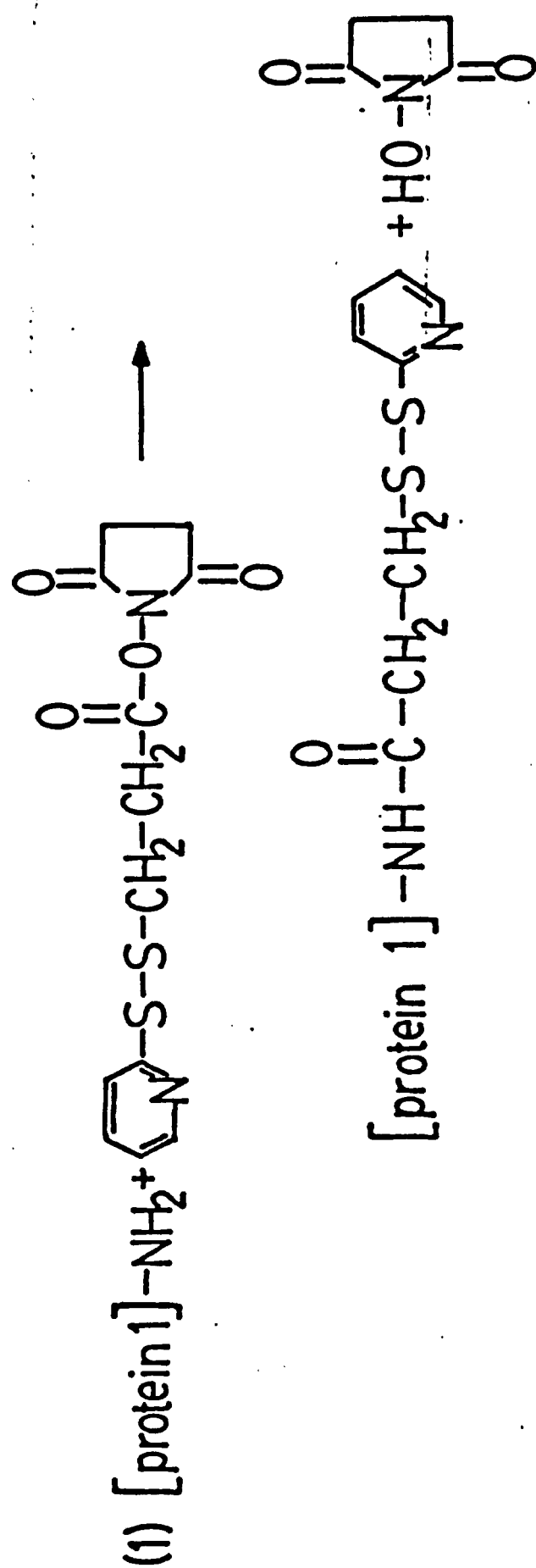
Scheme III: Application of the 2-thiopyridyl Group to Covalent Chromatography.

Carlsson and Svenson (22) reported the isolation of bovine mercaptalbumin by using the above method (Scheme III). Later, Carlsson et al. (23) showed a rapid and specific method for isolation of thiol-containing peptides from large proteins, for example, parvalbumin, bovine serum mercaptalbumin and ceruloplasmin, by thiol-disulfide exchange on a solid support.

Recently, reports illustrating the use of covalent chromatography to prepare enzymes and also the use of 2,2'-dipyridyl disulfide as a thiol titrant and a reactivity probe have appeared. For example, high-activity urease from Canavalia ensiformis (24) and fully active ficin from Ficus galbrata (25) are two systems which have been studied.

An analogous reagent n-propyl-2-pyridyl disulfide (Propyl-S-S-2-Py) reacts rapidly with thiol groups at the sulphur atom distal from the pyridyl ring with stoichiometric release of 2-thiopyridone. This suggests alkyl-2-pyridyl disulfide in general as valuable two-protonic-state reactivity probes with exceptional specificity for thiol groups (26). It also suggests reactions such as in Scheme II(2) are not occurring to any great extent. In 1978, Carlsson et al. (27) reported the heterobifunctional reagent N-succinimidyl 3-(2-pyridyldithio)propionate for preparation of protein-protein conjugates (Scheme IV).

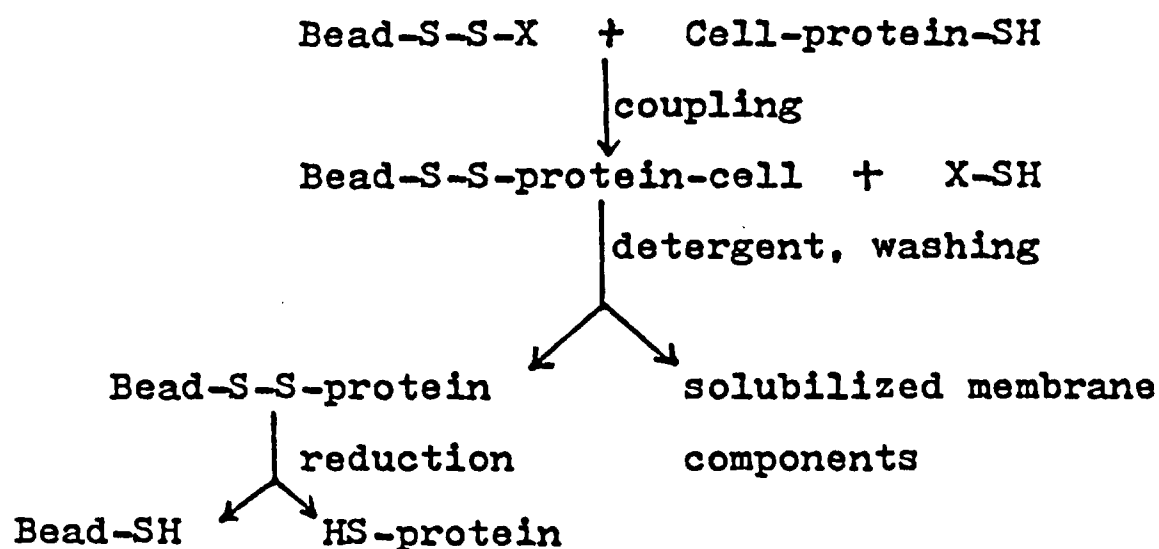
Affinity adsorbents for whole cells have been



Scheme IV : Protein-protein Conjugation Using N-succinimidyl 3-(2-pyridyldithio) Propionate.

developed based on covalent attachment of biospecific ligands such as antibodies, hormones or lectins to agarose beads (28). Covalent chromatography of cell membrane proteins via thiol-disulfide interchange has been reported previously (29) but in that case membrane components were solubilized in detergent prior to coupling with the agarose-based gels. In another report (30) an organomercurial-agarose matrix was used for rapid isolation of membrane glycoproteins, again after detergent solubilization.

The present study focuses on the development of bead reagents to be used in covalent chromatography of whole cells via thiol-disulfide interchange with surface components. The long-range goal is to assess the approach as a means of selective isolation of membrane proteins as depicted schematically below. In adapting covalent chromatography as above



to cell surface functional groups, endogenous or exogenous, several features of bead reagent design differing from those used for soluble proteins were considered:

- (a) Bead porosity may not be utilized and, indeed, could be a negative property. For chromatography of cells, none of the pore volume of the macroporous agarose gels would be available for coupling (4% agarose in bead form has an exclusion limit of  $20 \times 10^6$  Daltons) and, yet, the large preponderance of bead surface area and, hence, functional groups are located there. Within-pore functional groups could actually be a detriment to coupling as depicted above since uncoupled membrane components liberated upon detergent solubilization could couple at that stage and thereby be classed with the surface-exposed macromolecules.
- (b) Bead size becomes very important as a means of increasing surface area available for cell coupling without using large quantities of beads. This consideration is really a corollary to (a) above.
- (c) Bead-cell coupling represents a reaction between two solids, one of which, the cell is very "rough" on the molecular scale. Thus, it would seem advantageous to incorporate bead functional groups on very long, flexible spacer arms extended into bulk solvent, thereby allowing the reaction between two solids to be more like one between a surface reactant and a solution phase reactant.

Furthermore, the requirement that bead-anchored spacer arm extend into bulk solvent necessitates the choice of a hydrophilic oligomeric material for this component in contrast to the shorter hydrocarbon spacers used in many affinity chromatography applications.

Thus, more specifically, this study focuses on preparation and evaluation of a bead reagent capable of thiol-disulfide interchange reactions and with the following special features:

- (a) The bead matrix is essentially non-porous to biological macromolecules of interest.
- (b) Bead diameter is in the 10-37  $\mu\text{m}$  range which is 4-20-fold smaller than typically used in affinity chromatography.
- (c) Spacer arms are of the hydrophilic  $\alpha,\omega$ -bifunctional poly(oxyethylene) type whose average extended length would be 50-70 Å.

## CHAPTER II

### EXPERIMENTAL

#### A. Materials

The following materials were commercially available: Bovine serum albumin (BSA, Fatty acid-free), Iodoacetamide, 5,5'-dithio-bis-(2-nitrobenzoic acid)(DTNB), dithioerythritol (DTE), N-ethyl-5-phenyl-isoxazolium-3'-sulfonate (Reagent K) were from Sigma Chemical Company, St. Louis, MO. Papain (cryst.) was from Boehringer Mannheim GmbH. Chloroacetic acid, sodium carbonate, sodium acetate, ethylenediamine tetraacetate (EDTA), potassium hydroxide, potassium chloride, phosphorus pentoxide ( $P_2O_5$ ), sodium chloride, sodium hydroxide, hydrogen peroxide ( $H_2O_2$ ), sodium phosphate monobasic and dibasic, TRIS (hydroxymethyl)-aminomethane, dioxane, methanol, ethylenediamine, formamide, toluene (scintanalyzed) were from Fisher Scientific Company, Fair Lawn, N.J. Aldrithiol-2(2,2'-dipyridyl disulfide, 2-Py-S-S-2-Py), N-hydroxysuccinimide (NHS), triethylamine ( $Et_3N$ ) were from Aldrich Chemical Company, Milwaukee, WI. Dicyclohexylcarbodiimide (DCC), acetic anhydride ( $Ac_2O$ ) from BDH Chemicals Ltd., Poole, England. Coomassie G-250, Iodoacetic acid, 2,4,6-trinitrobenzene-sulfonic acid (TNBS), 2-mercaptoethanol were from Eastman Kodak Co., Rochester, N.Y. Acetic acid was from Allied Chemical Canada Ltd.,

Pointe Claire, Que. Hydrochloric acid was from J.T. Baker Chemical Co., Phillipsburg, N.J. Polyacrylamide gel P-2 (-400 mesh) was from BIO-RAD Laboratories, Richmond, CA. Sephadex G-25 medium (140-200 mesh) and Blue Dextran 2000 were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. [ $^{14}\text{C}$ ] -iodoacetamide (as an ethanol solution of specific activity 15.76 mcurie/mmole), Protosol and Omnifluor (98% of 2,5-diphenyloxazole and 2% of p-bis-[2-(5-phenyloxazoyl)] benzene) were obtained from New England Nuclear, Lachine, Que. Polyethylene glycol di(3-mercapto-propionate)(PEG-DMP) nominal average molecular weight 776 was obtained from Evans Chemetics, Darien, CT. Siliclad was obtained from Becton and Dickinson and Company, Parsippany, N.J. Potassium iodide was from Anachemia Chemicals Ltd., Montreal, Que. Membrane cones (Type CF 25) was obtained from Amicon Corporation, Lexington, MA. 2-acetamino-4-mercaptobutyric acid hydrazide (thiol hydrazide) was prepared and characterized by Y.C.Wu. of this lab (31).

## B. Equipment

All pH measurements were obtained on a Radiometer model 26 pH meter, equipped with a Radiometer semi-micro combination electrode GK2301-C. All aqueous solutions were prepared using distilled deionized water. Glassware that beads contacted was siliconized (using Siliclad) in order to



facilitate transfer, because polyacrylamide gel particles adhered strongly to glass surfaces.

All spectrophotometric measurements were performed on a Gilford model 2000 absorbance recorder attached to a Beckman DU monochromator or a Beckman model 35 spectrophotometer. Quartz Cells of 1-cm light path were used (1.2 or 3 ml volumes).

Scintillation counting was performed on a Nuclear Chicago Unilux II scintillation spectrometer equipped with a print out terminal. Centrifugation was performed on a Sorvall centrifuge RC-2-B in rotor SS34.

Sephadex G-25 medium was packed in 1.5x90 cm column K15 from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

## C. Methods

### 1. Analytical Methods

#### (a) TNBS Test (18)

TNBS solution was made 3% w/v in water. Samples are tested by mixing 4 ml of samples with 2 ml of saturated sodium borate, adding 3-5 drops TNBS solution, and comparing with a saline control after 5 minutes. After several minutes, primary amines give the colour ranging from yellow to orange red depending on the concentration of groups in the bead.

#### (b) Dry Weight Determination (18)

The polyacrylamide beads were added into a dry

Gooch Crucible whose weight was accurately known. It was washed twice by suction with 0.2 M sodium carbonate (for amino groups) to render the groups uncharged, then washed with distilled deionized water to remove electrolyte and finally washed with methanol. The material was allowed to dry in the vacuum desiccator over  $P_2O_5$  overnight. The crucible was weighed immediately after being removed from the desiccator.

(c) Determination of Aliphatic Amino Groups (18)

The bead sample was washed and resuspended (50% v/v) in 0.2 M KCl. The pH was adjusted to 11 with 1 M KOH, standard HCl (v, ml) of normality N was added to bring the pH to 7. The volume of suspension (V, ml) was measured. The amino group content was calculated using the following equation:

$$\text{meq.}(\text{NH}_2) = vN - (V - v) \times 0.001$$

(d) DTNB Titration of Beads

1 ml of a 50% (v/v) thiolated polyacrylamide beads suspension was reacted with 10 ml of 10 mM DTNB in 0.1 M sodium phosphate buffer, pH 8.0, containing 1 mM EDTA. The mixture was stirred for 15-30 minutes at room temperature. The supernatant was removed by filtration and the beads were washed with buffer several times. These beads were treated

with 10 ml of 15 mM DTE in 0.1 M sodium phosphate buffer, pH 8.0, containing 1mM EDTA for 15-30 minutes at room temperature. Then the filtrate was saved and united with a subsequent 5 ml buffer wash. The final volume was recorded. The content of the TNB<sup>6</sup> group released was calculated from the  $A_{412}$  using  $\epsilon_{412} = 1.36 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$  (32). The dry weight of beads was measured as above.

(e) Conversion of Beads to Bead-S-S-2-Py Form and 2-Py-S-S-2-Py Titration of Beads

2-Py-S-S-2-Py stock solution was prepared as follows (21): 40 mg of Aldrithiol-2(2-Py-S-S-2-Py) was added to 50 ml of water at room temperature and stirred magnetically for several hours. Insoluble material was then removed by filtration. This solution was stable for a month when stored at 4 C. Concentration, determined using  $\epsilon_{281} = 9.7 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$  (33), was in the range 1.1-1.4 mM.

The moist cake from 1 ml of 50% (v/v) thiolated bead suspension was suspended in 2.75 ml of 0.5 M sodium phosphate buffer, pH 7.55, containing 1 mM EDTA and 1.5 M KCl. This mixture was added in small portions to 10 ml 2-Py-S-S-2-Py stock solution and the final mixture was stirred for 20 minutes at room temperature. At the end of period, it was washed with buffer several times and then DTE back titration was carried out as for the above DTNB titration of beads. An  $\epsilon_{343}$  of  $8.08 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$  (34) was used to calculate the

concentration of 2-thiopyridone (Py-2-SH).

## 2. Alkylating Study

Polyethylene glycol di(3-mercaptopropionate)(PEG-DMP) reacts with iodoacetamide, iodoacetic acid, bromoacetic acid and chloroacetic acid individually in the following condition: 2 volumes of 5.7 mM (11.4 mN) PEG-DMP was combined with one volume of 10 mM or 20 mM above halo-compound and one volume of 0.1 M sodium phosphate buffer pH 8.6 or one volume of 10 mM or 100 mM KI at room temperature. At time intervals of 0.5, 1, 2, 4, 21, 24 hr., 50  $\mu$ l of the reaction mixture was taken separately and added to 3 ml of 0.1 mM DTNB solution which was dissolved in 0.1 M sodium phosphate buffer pH 8.6. Then it was measured by  $A_{412}$  versus a blank solution containing 50  $\mu$ l buffer and 3 ml of 0.1 mM DTNB solution.

## 3. Synthesis of the Long Arm Thiolated Polyacrylamide Beads

### (a) Preparation of Aminoethyl Polyacrylamide Beads (18)

In a fume hood, 10 g dry beads were added into 200 ml of ethylenediamine, which was preheated to 90°C, and stirred efficiently. The mixture was stirred gently for 20 minutes at 90°C. After 20 minutes, the suspension was mixed with an equal volume of crushed ice until the temperature cooled near room temperature. The beads were promptly washed with 0.2 M NaCl, 0.001 N HCl on a Büchner funnel and then

by batch washes until supernatants were negative to the TNBS test. The bead sample was washed twice and resuspended in 0.2 M KCl. The aminoethyl group content was measured by titration as described above.

(b) Preparation of Iodoacetamidoethylpolyacrylamide  
(Bead- $\overset{\text{O}}{\parallel}\text{C}\text{NH}\sim\text{NH}\overset{\text{O}}{\parallel}\text{C}\text{CH}_2\text{I}$ ) From Aminoethylated Polyacrylamide Beads  
(35)

The pH of aminoethylated polyacrylamide beads suspension (50% v/v) 60 ml was adjusted to 9.7-10.0 with dilute NaOH, then washed with water. Dicyclohexylcarbodiimide (4.4 g, 20 mmol) was dissolved in dry dioxane (40 ml). This solution was combined with an ice-cold solution of iodoacetic acid (3.7192 g, 20 mmol) and N-hydroxy-succinimide (2.36 g, 20 mmol) in dry dioxane (160 ml). The mixture was maintained in the dark and stirred gently for 1 hr at 0°C and 1 hr at room temperature. It was filtered, and the filtrate was added to the above suspension of aminoethylated polyacrylamide beads. The resulting suspension was stirred for 3 hours at room temperature and then it was filtered by using a Büchner funnel, washed with dioxane-water (1:2) twice, then with 1 M KCl and 0.2 M KCl. Finally it was stored in 0.2 M KCl. Aminoethyl group densities were measured as described above.

(c) Blocking Unreacted Amino Group of Iodoacetamido-ethylpolyacrylamide Beads

10 g dry iodoacetamidoethylpolyacrylamide beads,

containing around 50  $\mu$ eq.  $\text{NH}_2$  group was combined with a solution of acetic anhydride (1.134 ml. 12 mmol) and triethylamine (2 eq. amt., 13.93  $\mu$ l) in formamide (40 ml). The mixture was stirred for 3 hours at room temperature and then was added to 40 ml  $\text{H}_2\text{O}$  and stirred for 15-30 minutes. The beads were filtered, washed with 1 M KCl and 0.2 M KCl and stored in 0.2 M KCl. Aminoethyl group densities were checked as usual.

(d) Preparation of Long Arm Thiolated Beads (Bead-  
 $\text{O} \quad \text{O} \quad \text{O}$   
 $\text{C} \text{NHCH}_2\text{CH}_2\text{NH} \text{C} \text{CH}_2\text{SCH}_2\text{CH}_2 \text{C} \text{O} (\text{CH}_2\text{CH}_2\text{O})_n \text{C} \text{CH}_2\text{CH}_2\text{SH} \equiv \text{Bead-SH}$ ) from  
 Iodoacetamidoethylpolyacrylamide Beads

10 g dry iodoacetamidoacetyl polyacrylamide beads were added into a solution of polyethylene glycol di(3-mercapto-propionate)  $\text{HSCH}_2\text{CH}_2 \text{C} \text{O} (\text{CH}_2\text{CH}_2\text{O})_n \text{C} \text{CH}_2\text{CH}_2\text{SH}$  (40-50 eq. dithiol by DTNB titration; average molecular weight 683 by this titration) in 0.1 M sodium phosphate buffer (70 ml), pH 8.6, containing 1 mM EDTA. The mixture was stirred for 24 hours at room temperature before treatment with 2-mercapto-ethanol at a final concentration of 100 mM, and stirring for an additional 24 hours at room temperature. The supernatant was removed and the beads were washed with water containing 5 mM DTE twice, then with 1 M KCl containing 5 mM DTE and 0.2 M KCl containing 5 mM DTE. The preparation was stored in the latter solution at 4°C. Thiol content was determined by DTNB titration and 2-Py-S-S-2-Py titration.

#### 4. [ $^{14}\text{C}$ ] -Iodoacetamide Coupling with Bead-SH

Two samples were prepared as follows: 0.2 ml of 50% (v/v) beads suspension which was treated with 1 mM DTE in 0.1 M sodium phosphate buffer pH 7.5 containing 1 mM EDTA. The samples were transferred to tared 10 x 75 mm tubes and washed with buffer by centrifugation. The first sample was treated with non-radioactive iodoacetamide first, and washed twice with buffer. Then both tubes were treated with labeled iodoacetamide and left for 1 hour at room temperature. After this, each tube was washed with buffer several times and then with methanol twice by centrifugation. Bead samples were dried in a vacuum desiccator over  $\text{P}_2\text{O}_5$  and weighed. 0.4 ml of 30%  $\text{H}_2\text{O}_2$  was added to each tube, the tubes were sealed and placed in an oven (70-75°C) until the beads were dissolved. After 3-4 days, the sealed sample tubes were taken from the oven and kept at -20°C overnight. The sample tubes were immersed in liquid nitrogen and opened. (Caution: failure to cool these tubes results in a violent release of pressure upon opening). Samples were transferred individually into scintillation vials by adding 1.5 ml Protosol, each in small portions, followed by 1 ml cocktail. Each scintillation vial finally was diluted with 10 ml cocktail, mixed well and counted. The cocktail used here was Omnifluor (4 g/l) in toluene. Sample counting efficiency was determined by the channels ratio method using a set of  $^{14}\text{C}$  quenched standards

from Nuclear Chicago. A typical quench correction curve is given in the Appendix 1.

## 5. Papain Coupling with Bead-S-S-2-Py

### (a) Preparation of Activated Papain (36)

Papain (suspension of 20 mg in 2 ml 50 mM- sodium acetate buffer pH 4.5) was adjusted pH to 8.0 with 1 M TRIS stock. To this solution was added deionized water until the papain dissolved. Then the papain solution was activated by treatment with 0.4 ml of 100 mM DTE (final DTE concentration 5 mM) for 15 minutes and the activator removed by passage through a Sephadex G-25 column.  $N_2$ -saturated 0.5 M sodium acetate buffer pH 4.5 containing 0.15 M NaCl and 1 mM EDTA was used throughout. The desired fractions were pooled on the basis of  $A_{280}$  measurements. The molecular weight of papain was taken as 23,000 (37) and  $E_{278}^{1\%}$  for papain as 25 (38). Thiol content of the papain was determined by titration with DTNB and 2-Py-S-S-2-Py.

### (b) Activated Papain Coupling with Bead-S-S-2-Py

Bead-S-S-2-Py was made as above. The moist cake from 4 ml of 50% (v/v) bead-S-S-2-Py suspension in 25 ml Gooch Crucible was mixed with 6 ml of activated papain obtained from the Sephadex G-25 column. The mixture was stirred gently for 20 minutes at room temperature, then filtered and washed with the acetate buffer.  $A_{278}$  and  $A_{343}$  of the filtrate



were measured, and thiol and R-S-S-2-Py contents of the filtrate were determined by 2-Py-S-S-2-Py and DTE titration, respectively. The beads were treated with 15 mM DTE (in 0.1 M sodium phosphate buffer, pH 7.5 with 1 mM EDTA) back titration. Then the filtrate was dialyzed over two nights against several changes of N<sub>2</sub>-saturated acetate buffer of pH 4.5. After dialysis, the resulting solution was characterized by A<sub>278</sub> and by titration with 2-Py-S-S-2-Py at pH 4.5 and pH 8.0.

#### 6. BSA Coupling with Bead-SH

400 mg of BSA was dissolved in 2 ml of 0.1 M sodium phosphate buffer, pH 7.55, containing 1 mM EDTA and 0.3 M KCl. This BSA solution, which was treated with 0.9 eq. of DTE (353  $\mu$ l, 15 mM DTE) for 30 minutes at room temperature was isolated from a Sephadex G-25 column. The thiol content was 0.8-1.0 SH per unit by 2-Py-S-S-2-Py titration.

##### (a) BSA Coupling with Bead-S-S-2-Py

Bead-S-S-2-Py was made as above. Two samples were prepared as follows: The moist cake from 4 ml of 50% bead-S-S-2-Py (in 0.2 M KCl) suspension in a 25 ml Gooch Crucible was mixed with 1.5 ml of BSA-SH obtained from Sephadex G-25 column and 2 ml of 0.05 M sodium acetate buffer, pH 4.5, containing 1 mM EDTA and 0.15 M NaCl. The two samples were stirred separately for 1 minute and 10 minutes at room

temperature and then filtered and washed with the acetate buffer. Values of  $A_{278}$  and  $A_{343}$  of the filtrates were measured. The thiol and R-S-S-2-Py contents of the filtrate were determined by titration of 2-Py-S-S-2-Py and DTE respectively. The molecular weight of BSA was taken as 68,000 and  $E_{278}^{1\%}$ ,  $E_{280}^{1\%}$  for BSA as 6.7, 6.6 (22,39).

For many experiments, BSA was used directly as obtained (approx. 0.4 eq. SH/mole) in 0.1 M sodium phosphate pH 7.55 containing 1 mM EDTA.

For the long coupling reaction times (3-16 hr.), all the procedures were same as described above. But protein was determined from  $A_{280}$  measurements on the supernatant solutions which had been concentrated and washed free of Py-2-SH in a ultrafiltration centrifuge cone apparatus. This cone apparatus, using a type CF-25 membrane, was centrifuged <2750 rpm for 15 min. The other assay for protein was by using the Coomassie Brilliant blue G-250 method of Bradford (40).

#### (b) BSA-S-S-2-Py Coupling with Bead-SH

BSA-S-S-2-Py was made as follows: Dropwise addition of 2 ml of BSA-SH from Sephadex G-25 filtration to 2 ml of 2-Py-S-S-2-Py stock solution was followed by stirring for 1 hour at room temperature. BSA-S-S-2-Py solution was isolated from Sephadex G-25 column. 0.05 M sodium acetate buffer, pH 4.5, containing 1 mM EDTA and 0.15 M NaCl was

used throughout. The desired fractions were collected on the basis of  $A_{278}$  measurements. BSA-S-S-2-Py content was determined by titration with DTE.

1 ml of 50% (v/v) bead-SH ( $0.5-1.0 \mu\text{mole-SH}$ ) was washed with  $N_2$ -saturated acetate buffer, pH 4.5, and treated with 6 ml of BSA-S-S-2-Py ( $0.066-0.184 \mu\text{moles-S-S-2-Py}$ ) obtained as above for 10 min. at room temperature. The washing step, DTE cleavage step, and protein determination were as described above.

## 7. Cell Coupling with Bead-S-S-2-Py

The human erythrocyte membrane (ghost) preparations were carried out by the method of hypotonic lysis as summarized by Steck (41). The final preparation consisted of a 50% suspension in 0.1 M sodium phosphate buffer, pH 7.5.

### (a) Ghosts Reaction with 2-Acetamino-4-mercaptobutyric Acid Hydrazide (Thiol Hydrazide)

A ghost sample ( $\sim 20$  ml, 50% suspension) was treated with 10 mM DTE for 20 min at room temperature. Then it was centrifuged at 10,000-12,000 rpm for 10 min, the supernatant was discarded and the packed ghosts were diluted to the initial volume with 40 mM iodoacetamide and maintained at room temperature for 30 min. The ghost sample was washed by centrifugation with 0.1 M sodium phosphate buffer, pH 7.5, then with PBS once and resuspended in PBS. 1 ml of this

50% ghost suspension was taken for 2-Py-S-S-2-Py titration and 0.1 ml for protein assay as below. The remainder of the 50% ghost suspension was treated with 3.34 ml of 0.02 M  $\text{NaIO}_4$  in PBS for 15 min at room temperature. Then it was centrifuged, washed twice with PBS, resuspended in the same and treated with 6.66 ml of 0.01 M thiol hydrazide in PBS. After a 2 hr incubation at room temperature, the mixture was diluted with ~10 ml of PBS, centrifuged and washed twice with 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA. Consequently a 50% suspension in above buffer was prepared. The thiol content was determined by 2-Py-S-S-2-Py titration in the absence of SDS. Protein was determined by using the coomassie brilliant blue G-250 procedure as described by Bradford (40).

(b) Ghost-Hydrazone Coupling with Bead-S-S-2-Py

Seven milliliters of ghost-hydrazone (50% suspension) was added to 5 ml 50% bead-S-S-2-Py, which was maintained at room temperature for 30 min. It was then centrifuged at 12,000 rpm for 5 min. The coupling supernatant was characterized by its  $A_{343}$  and protein assay. The residue was washed twice by centrifugation at 500 rpm for less than 1 min in 0.1 M sodium phosphate buffer, pH 7.5, containing 1 mM EDTA.

The bead residue was then washed once with 1% SDS containing 20 mM iodoacetamide and then washed twice

with 0.1% SDS in buffer. The washed residue was finally treated with 4 ml of 15 mM DTE in 0.1% SDS for 20-30 min at room temperature. The supernatant was kept for protein determination as described above.

## 8. SDS Polyacrylamide Gel

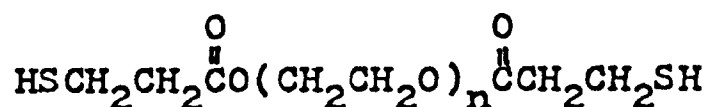
The gel was prepared as described by Weber and Osboron (42), except that Pyronin G was used as the tracking dye. After electrophoresis the gels were stained with coomassie blue G-250 and cleared as described by Fairbanks, Steck & Wallach (41). They were then scanned with an Ortec 4310 densitometer at 550 nm.

## CHAPTER III

### RESULTS

#### A. Preparation of Long-Arm Thiolated Polyacrylamide Beads

The long-arm spacer molecule tested in this study was a sample of polyoxyethylene glycol di(3-mercaptopropionate), PEG-DMP, of general formula:



The material obtained from Evan's Chemetics was of nominal average molecular weight 776 (i.e.  $n=13.2$ ). Spectrophotometric titration of the thiol content using DTNB and/ or 2-Py-S-S-2-Py indicated an average molecular weight of 683. The overall plan for incorporation of this material into a bead material is shown in Scheme V. Commercially obtained polyacrylamide beads was Bio-Gel P-2 (~400 mesh), i.e. particle diameter 10-37  $\mu\text{m}$  and inclusion limit (molecular weight cutoff) ~1800 Daltons.

##### 1. Alkylation Study

A model study using the poly(oxyethylene)- $\alpha,\omega$ -dithiol and a selection of alkylating agents was carried out in order to determine approximate conditions for the bead

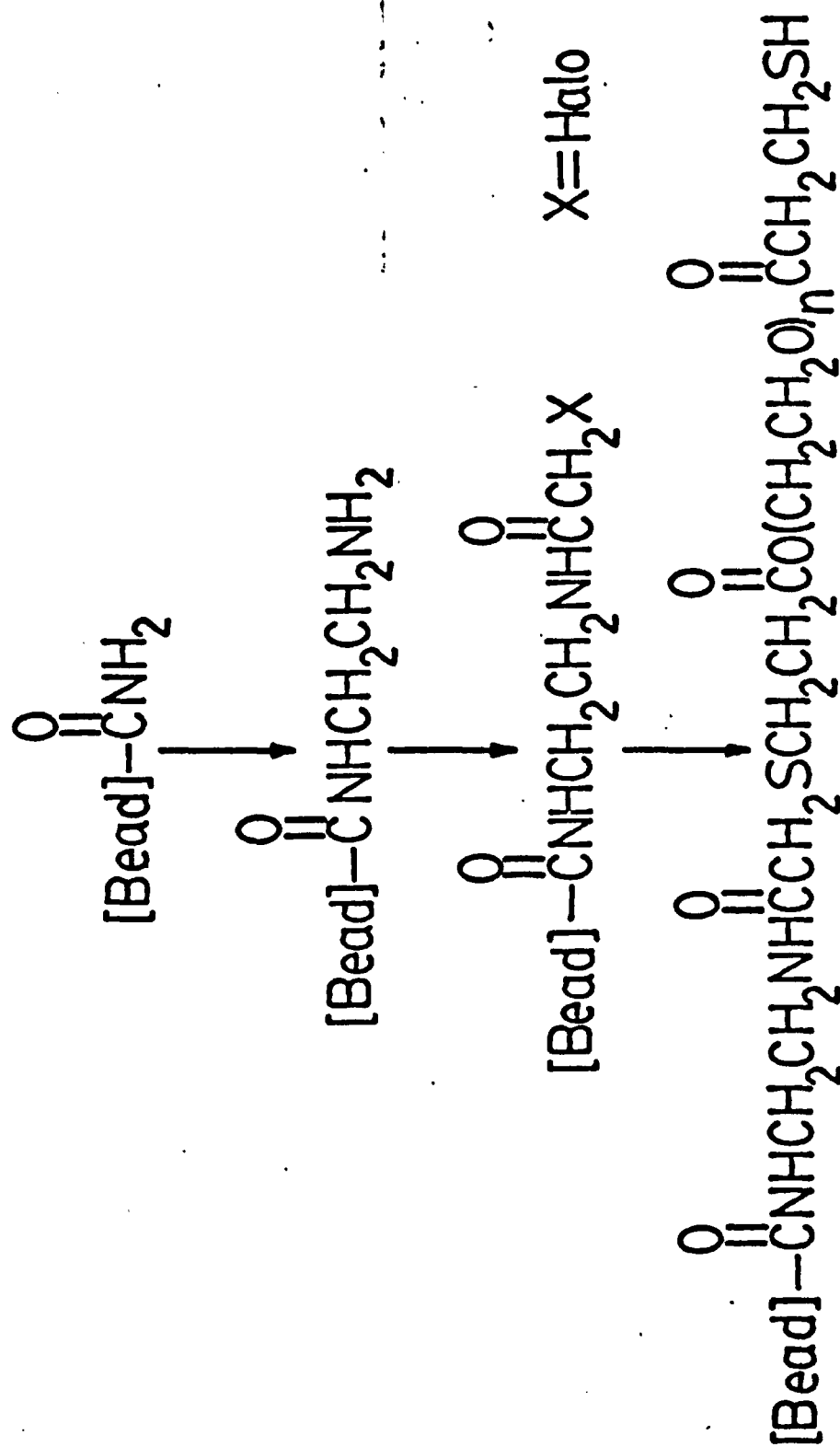
alkylation step of Scheme V. Reactions were monitored by spectrophotometric titration of the thiol remaining in solution as a function of time. Conditions and results are given in Table I. From these results it was decided that iodo- or bromo-acetyl groups would be tested in the bead synthesis outlined in Scheme V.

## 2. Aminoethylpolyacrylamide Beads

Aminoethylation of polyacrylamide beads with ethylene diamine at 90°C was carried out as described by Inman (18). For heating times of 20, 30 and 45 minutes, the amino group titres were respectively  $52 \pm 5$  (4 determinations), 78 (one determination) and  $174 \pm 10$  (2 determinations)  $\mu\text{eq./g}$  of dry beads.

## 3. Haloacetamidoethylpolyacrylamide Beads

A number of methods common in peptide chemistry were evaluated for coupling of haloacetic acids to aminoethylpolyacrylamide beads. The coupling survey was initiated with chloroacetic acid using (a) water soluble carbodiimide 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide (EDAC; Scheme VI) (43); (b) dicyclohexylcarbodiimide (DCC) and N-hydroxy-succinimide (NHS) to form an intermediate chloroacetyl-NHS ester which was allowed to acylate aminoethyl beads (Scheme VII) (35) and (c) N-ethyl-5-phenyl-isoxazolium-3'-sulfonate



Scheme V : Plan for Construction of Long-arm Thiolated Poly-acrylamide Beads.



Table I: Alkylating Study with Polyethylene Glycol Di(3-mercaptopropionate)<sup>a</sup>

PEG-DMP <sup>c</sup> (mN)	Reagents					Residual Thiol Concentration(mM) <sup>b</sup>					
	ICH <sub>2</sub> CONH <sub>2</sub> (mM)	ICH <sub>2</sub> COOH (mM)	BrCH <sub>2</sub> COOH (mM)	ClCH <sub>2</sub> COOH (mM)	KI (mM)	$\frac{1}{2}$ hr.	1 hr.	2 hr.	4 hr.	21 hr.	24 hr.
5.7	2.5	-	-	-	-	1.7	1.7	1.8	-	-	-
5.0	5.0	-	-	-	-	0.02	0	-	-	-	-
5.0	-	5.0	-	-	-	2.6	2.0	1.2	0.64	0	-
5.0	-	-	5.0	-	-	3.6	3.0	2.2	1.3	0.02	-
5.0	-	-	5.0	-	25	3.5	2.7	2.0	1.1	0	-
5.0	-	-	-	-	-	5.0	4.8	4.6	4.4	2.7	-
5.7	-	-	-	2.5	-	5.5	5.6	5.6	5.6	3.8	3.6
5.7	-	-	-	2.5	25	5.0	5.6	5.6	5.6	3.7	3.5
5.7	-	-	-	-	-	5.0	5.6	5.6	5.9	3.5	3.3

a. Reagents were incubated in 0.1 M sodium phosphate buffer of pH 8.6 at room temperature.

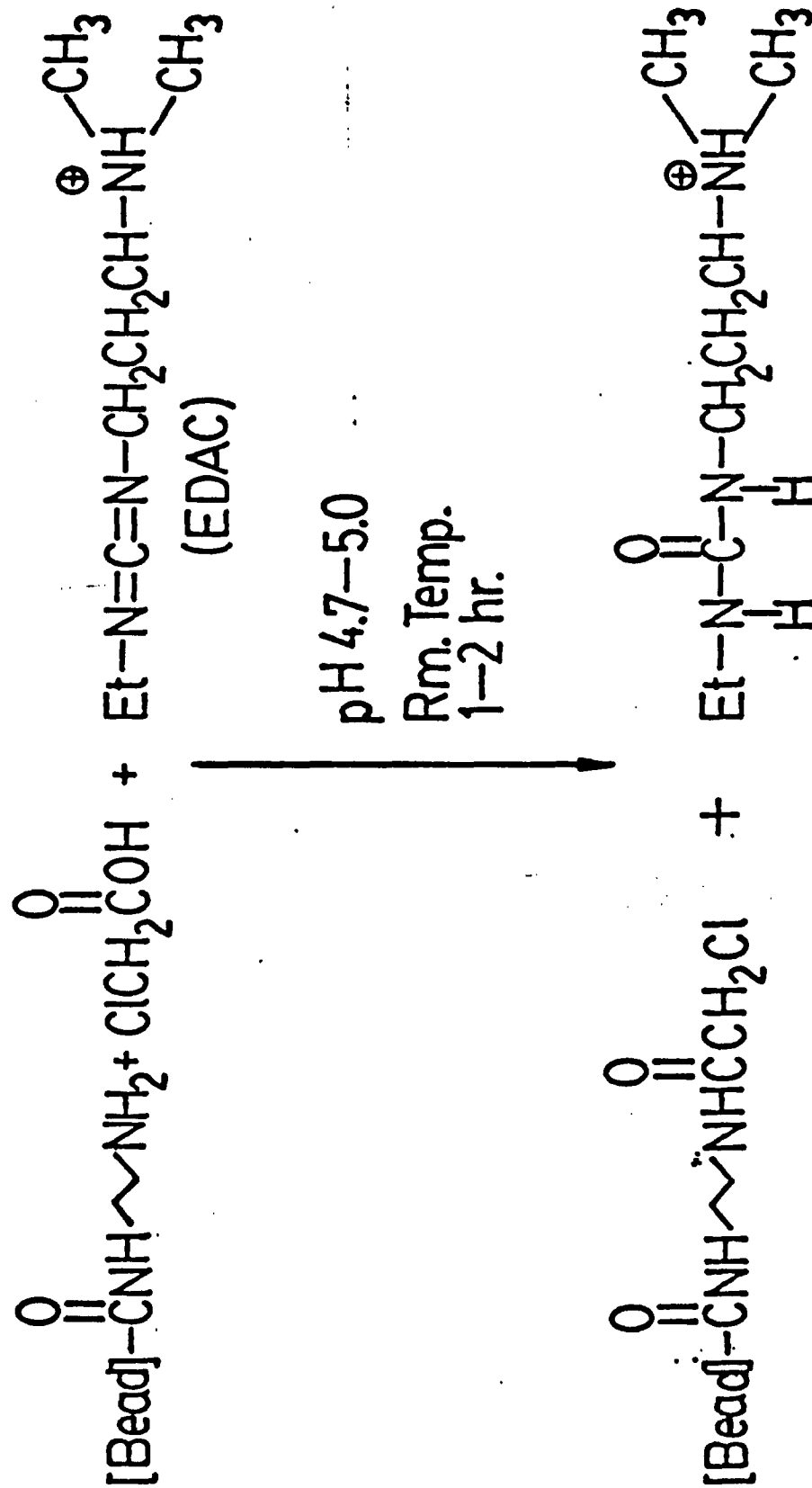
b. Residual thiol concentrations were determined on aliquots of the reaction mixture at the times indicated. The aliquots were diluted 61-fold with DTNB stock solution (DTNB

**Table I: Alkylating Study with Polyethylene Glycol Di(3-mercaptopropionate)<sup>a</sup>**

(Cont'd)

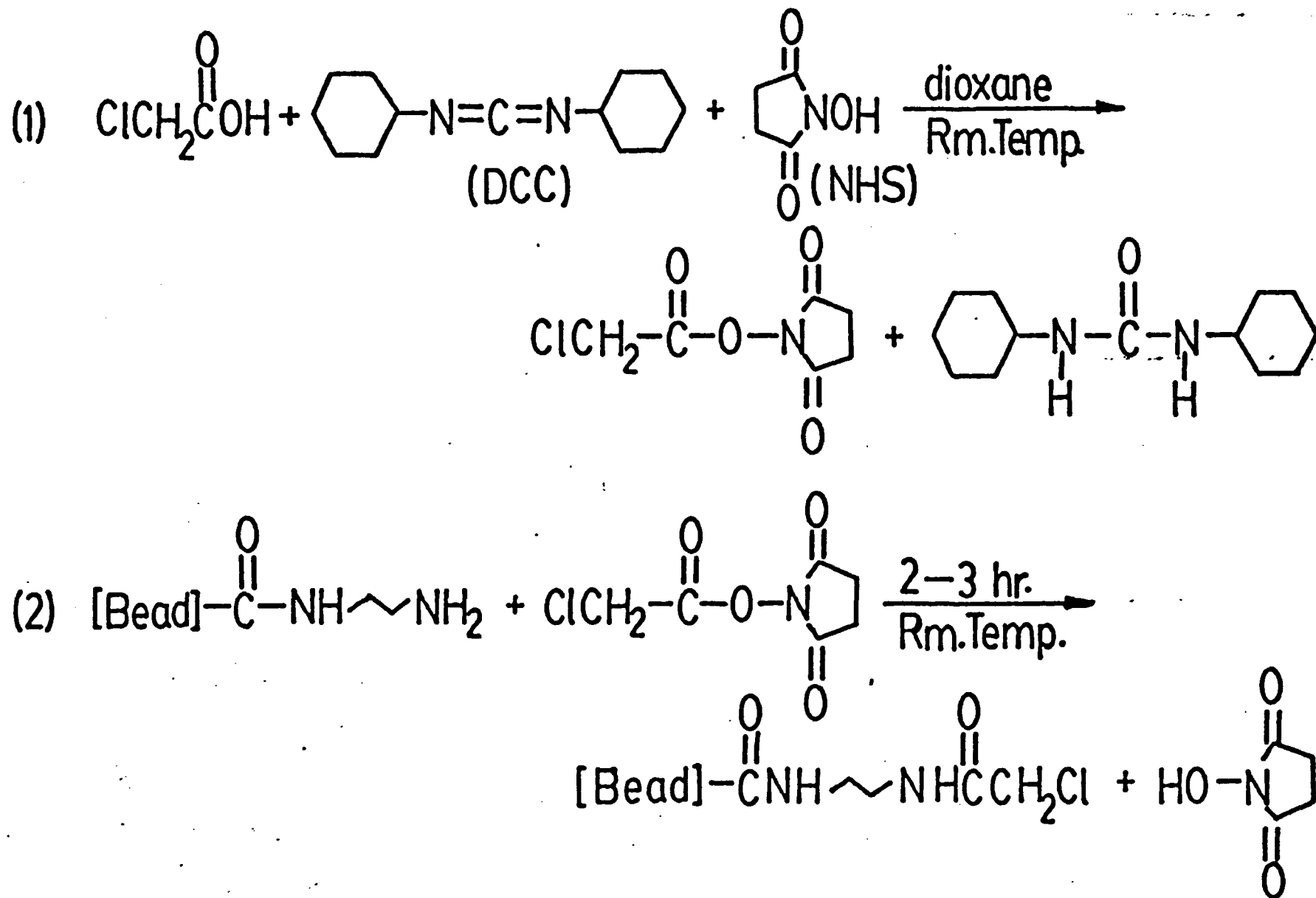
final concentration of 0.1 mM DTNB) in the same buffer and  $A_{412}$  was read after 20 min.

c. PEG-DMP is polyethylene glycol di(3-mercaptopropionate).



Scheme VI: Chloroacetylation of Aminoethyl Polyacrylamide Beads

Using 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide(EDAC).



Scheme VII: Chloroacetylation of Aminoethyl Polyacrylamide Beads Using Dicyclohexylcarbodiimide(DCC) and N-hydroxy-succinimide(NHS).

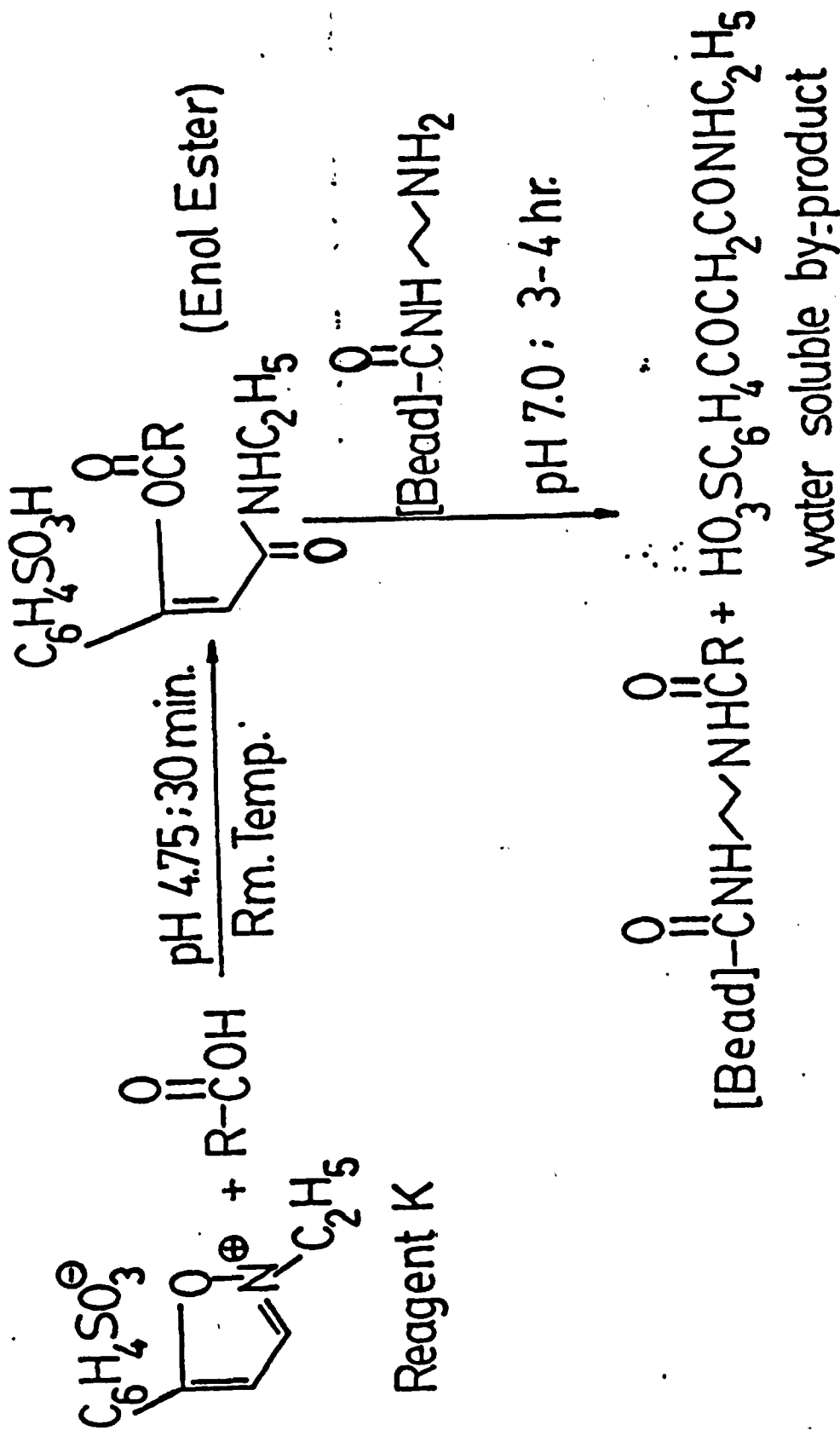
(Reagent K; Scheme VIII) (44). Results are given in Table II. All subsequent preparations were carried out with iodoacetylation using DCC and NHS.

The foregoing results show that quantitative conversion of all bead aminoethyl groups to haloacetamidoethyl groups was not possible. For this reason an acetylation step was included in the synthetic pathway in an attempt to completely mask unreacted amino groups. Suspension of dry beads in formamide and treatment with acetic anhydride plus triethylamine followed by aqueous workup and amino group titration indicated acylation of 85-95% of the remaining aminoethyl sites had occurred. For example, amino group titres of 25 and 26  $\mu\text{eq./g}$  before treatment, became 4.4 and 5.8  $\mu\text{eq./g}$ , afterward.

#### 4. Alkylation of $\alpha,\omega$ -dithiols using Haloacetamidoethyl Beads

Thioether formation between haloacetamidoethyl beads and the "long arm" dithiol, PEG-DMP, was attempted using a 20-fold molar excess of the latter in order to favour mono-alkylation. The anticipated reaction is presented in Scheme IX (part a) along with conditions. Degree of coupling was inferred from spectrophotometric titration of thiol groups on the bead samples. Results are given in Table III.

Under the above conditions a "short arm" dithiol



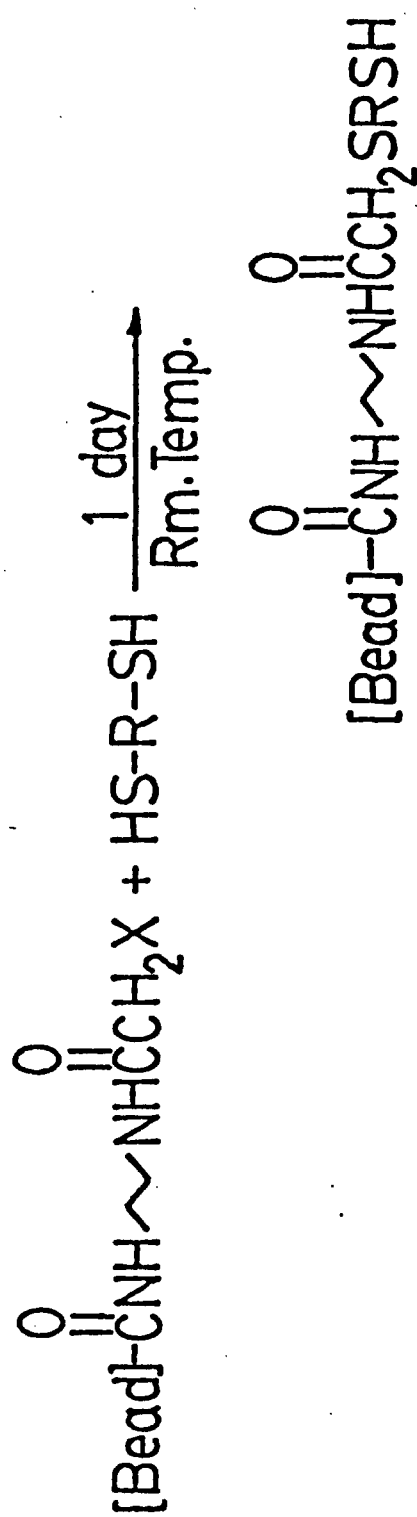
Scheme VIII :Chloroacetylation of Aminoethyl Polyacrylamide Beads

Using N-ethyl-5-phenyl-isoxazolium-3'-sulfonate(Reagent K).

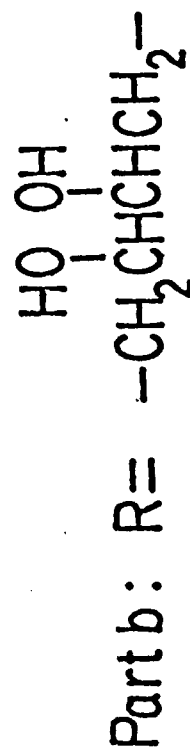
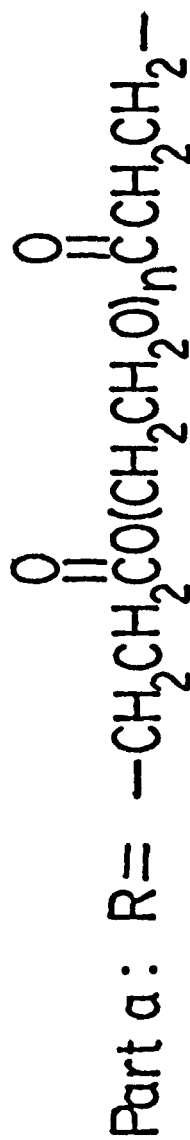
Table II: Coupling Aminoethyl Polyacrylamide  
Beads and Haloacetic Acid <sup>a</sup>

$\text{Bead}-\overset{\text{O}}{\parallel}\text{CNH}\sim\text{NH}_2 + \text{XCH}_2\overset{\text{O}}{\parallel}\text{COH}$ Reagent	Before reaction original-NH <sub>2</sub> groups( $\mu\text{eq.}/\text{g}$ )	After reaction unreacted-NH <sub>2</sub> groups( $\mu\text{eq.}/\text{g}$ )	Haloacetyl groups bound ( $\mu\text{eq.}/\text{g}$ )	% of halo- acetyl group bound
EDAC <sup>b</sup> + ClCH <sub>2</sub> COOH	77.7 $\pm$ 1.6	50.8 $\pm$ 3.0	26.9 $\pm$ 4.6	35.0 $\pm$ 7.0
Reagent K + ClCH <sub>2</sub> COOH	77.7 $\pm$ 1.6	30.8 $\pm$ 0.4	46.9 $\pm$ 2.0	60.0 $\pm$ 4.0
DCC + NHS + ClCH <sub>2</sub> COOH	183.5 $\pm$ 0.5	62.6 $\pm$ 3.8	120.9 $\pm$ 4.3	66.0 $\pm$ 2.5
DCC + NHS + BrCH <sub>2</sub> COOH	164.0	78.2	85.8	52.0
DCC + NHS + ICH <sub>2</sub> COOH	164.0	62.8	101.2	62.0

- a. Conditions of coupling are given in Schemes VI, VII and VIII. Coupling was estimated from the decrease in amino group titre of the bead samples. In each reaction with a 10-20-fold excess of acylating reagent over bead amino groups.
- b. EDAC is 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide. DCC is Dicyclohexyl-carbodiimide, NHS is N-hydroxy-succinimide, Reagent K is N-ethyl-5-phenyl-isoxazolium-3'-sulfonate.



X = Cl, Br or I



Scheme IX: Reaction of Haloacetamidoethylpolyacrylamide with Dithiols



Table III: Reaction of Haloacetamidoethylpolyacrylamide with polyethylene glycol di(3-mercaptopropionate)

Reagent	Rx. Time	Before Rx. Haloacetyl Group Content <sup>b</sup> ( $\mu\text{mol/g}$ )	After Rx. Thiol Group Content <sup>c</sup> ( $\mu\text{mol/g}$ )	Thiol Group content as % of Sites available.
Bead-COCH <sub>2</sub> Cl <sup>a</sup> +KI	1 day	120.9	8.5	7.0
Bead-COCH <sub>2</sub> Br <sup>a</sup>	1 day	85.8	12.6	14.7
	4 days	85.8	11.7	13.6
Bead-COCH <sub>2</sub> I <sup>a</sup>	1 day	101.2	13.8	13.6
	4 days	101.2	12.2	12.1

a. The symbols bead-COCH<sub>2</sub>Cl, bead-COCH<sub>2</sub>Br and Bead-COCH<sub>2</sub>I represent Chloro-, Bromo- and Iodo-acetamidoethylpolyacrylamide.

b. Haloacetyl group content was inferred from the decrease in amino group titration

c. Thiol group content was determined by spectrophotometric titration using DTNB.

dithio-erythritol (DTE), was coupled to some of the haloacetamidoethyl bead preparations. The anticipated reaction is indicated by part b of Scheme IX and comparisons of reaction of long- and short-arm dithiols to the same bead sample are given in Table IV. The results clearly indicate that there are haloacetamidoethyl groups on the beads which are not accessible to PEG-DMP but which do react with the smaller molecule, DTE. With the aim of blocking some of these unreacted haloacetamido groups coupling of long arm dithiol to beads was routinely followed by treatment with a high concentration of 2-mercaptoethanol in the same coupling buffer.

## 5. Summary of Overall Synthesis of Long-Arm Thiolated Beads

The preferred pathway for preparation of long-arm thiol beads is given in Scheme X and Table V presents the results of three preparations which were taken through the steps of Scheme X.

## B. Characterization of Long-Arm Thiolated Beads in Thiol-Disulfide Interchange Reactions

For reasons given in the Introduction thiol-disulfide interchange reactions studied here all involved an "activated" disulfide, ones which upon reaction with a thiol resulted in expulsion of an aromatic thiol(ate). Two

Table IV: Comparison of Short Arm and Long Arm Thiolated Beads <sup>a</sup>

	Bead-COCH <sub>2</sub> I with		Bead-COCH <sub>2</sub> Br with		Bead-COCH <sub>2</sub> I with	
	PEG-DMP	DTE	PEG-DMP	DTE	PEG-DMP	DTE
Before reaction, haloacetyl group content <sup>b</sup> (μmol/g)	101.2	101.2	85.8	85.8	30.3	30.3
After reaction, thiol group content <sup>c</sup> (μmol/g)	13.8	28.9	12.6	28.2	4.6	7.8
Thiol group content as % of sites available	13.6	28.6	14.7	32.9	15.2	25.7
Short arm thiol/ long arm thiol	2.1-fold		2.2-fold		1.7-fold	

a. PEG-DMP is polyethylene glycol di(3-mercaptopropionate). DTE is Dithioerythritol. The symbols bead-COCH<sub>2</sub>I and bead-COCH<sub>2</sub>Br represent the corresponding haloacetamido-ethyl bead preparations. In all cases 20 moles of thiol were allowed to react with bead haloacetamido groups at room temperature in pH 8.6 suspension for 24 hours.

b. Haloacetyl group content was estimated indirectly from amino group titrations as in Table III.

c. Thiol content was determined by spectrophotometric titration using DTNB.



Table V: Synthesis of Long Arm Thiolated Beads from Polyacrylamide Beads

	Expt. 1	Expt. 2	Expt. 3
Bead-CONH~NH <sub>2</sub> <sup>a</sup> (μeq./g NH <sub>2</sub> gr.)	47	49	48
Bead-CONH~NHCOCH <sub>2</sub> I <sup>b</sup> (μeq./g CH <sub>2</sub> I.)	26 (56%)	44 (90%)	25 (52%)
Bead- $\begin{matrix} \text{CONH~NHCOCH}_2\text{I} \\ \text{CONH~NHAc} \end{matrix}$ <sup>c</sup> (μeq./g NH <sub>2</sub> gr.)	5.8(88%)	0 (100%)	4.4(91%)
Bead-SH <sup>d</sup> (μmol/g)	5.0(19%)	5.8(13%)	3.2(13%)

a. Amino group content was determined by potentiometric titration.

b. Iodoacetamido group content was inferred from the decrease in amino group titration (percentage amino groups lost).

c. Acetamido group content was inferred from the additional decrease in amino group titration (percentage of remaining amino groups lost).

d. Thiol group content was determined by spectrophotometric titration using DTNB (percentage of iodoacetamido groups alkylated).

Schemes were considered:



where the aryl moiety was derived from 2-mercaptopyridine (Py-2-SH) or 5-mercapto-2-nitrobenzoic acid (thionitrobenzoate, TNB).

### 1. Activated Thiolated Beads, Bead-S-S-Ar

Long-arm thiolated beads were converted into the activated mixed disulfide form simply by scaling up the first stage of thiol determination using 2-Py-S-S-2-Py or DTNB. Thus, bead-SH suspension was added slowly with thorough mixing to a large excess of one of the latter reagents followed by exhaustive washing with buffer and finally with 0.2 M KCl. Beads were usually stored in the latter medium at 4°C. Small aliquots were characterized by back titration with DTE.

### 2. Accessibility of Bead-S-S-2-Py to Small Thiols

Free PEG-DMP was used as a probe of accessibility of bead-S-S-2-Py sites. Interchange reactions carried out at room temperature and pH 7.55 for 3 hours with 1.7, 2.8 and 4.4 equivalents of thiol per equivalent of bead-S-S-2-Py respectively showed release of 89, 94 and 88% of the available

2-thiopyridone (where "available" Py-2-SH was that released by 15 mM DTE at pH 7.55 after 30 minutes at room temperature).

2-mercaptoethanol was used as a small kinetic probe of bead-S-S-2-Py groups. In this case an approximately 20-fold excess of bead-S-S-2-Py was allowed to react with the thiol at pH 7.55 for short periods with the results given in Table VI.

### 3. [ $^{14}\text{C}$ ] -Iodoacetamide Alkylation of Bead-SH

As an independent check of bead thiol group density, radio-chemical titration was carried out using [ $^{14}\text{C}$ ] -iodoacetamide of known specific activity. Samples from two preparations, freshly treated with 10 mM DTE and finally centrifuged once in 1 mM DTE, were mixed in thick suspension with excess labeling reagent, with and without pre-treatment with non-radioactive reagent. After copious washing with aqueous buffer and then methanol, bead samples were dried and weighed portions were digested in 30% hydrogen peroxide prior to liquid scintillation counting in a toluene-based cocktail. Quench correction was carried out using the channels ratio method based on a set of [ $^{14}\text{C}$ ] -standards from the instrument manufacturer (a typical quench correction curve is given in Appendix 1). Counting efficiencies for these samples were 82-89% and results are given in Table VII.

Table VI: 2-mercaptoethanol Coupling with Bead-S-S-2-Py <sup>a</sup>

2-mercaptoethanol (0.1324 $\mu$ mol) Coupling with	Reaction Time(min.)	Py-2-SH Released ( $\mu$ mol)	% of 2-mercapto- ethanol Bound
Bead-S-S-2-Py (2.92 $\mu$ mol)	1-2	0.068	51
Bead-S-S-2-Py (2.5 $\mu$ mol)	10	0.092	70

a. The quantities indicated were combined in approximately 40% bead suspension at room temp. in pH 7.55 buffer. At the times indicated the suspensions were rapidly filtered, beads were washed, the wash was combined with the first filtrate and  $A_{343}$  of the filtrate was measured.



Table VII: [ $^{14}\text{C}$ ]-Iodoacetamide Coupling with Bead-SH <sup>a</sup>

	Sample			
	1	2	3	4
dpm in sample	4,353	220,186	1,681	118,192
$\mu\text{mol}$ [ $^{14}\text{C}$ ] -carboxamidomethyl/g	0.075	3.6	0.024	2.0
$\mu\text{mol}$ bead-SH/g from 2-Py-S-S-2-Py titration	8.4	8.4	5.0	5.0
[ $^{14}\text{C}$ ]-carboxamidomethyl incorporated as a percentage of bead-SH	0.9	43	0.5	40

a. Bead samples (corresponding to ca 0.029 g dry beads) as packed suspensions in 0.1 M sodium phosphate buffer of pH 7.5 in the presence of 1 mM DTE were treated with iodoacetamide at room temperature at an approximate final concentration of 15 mM (57% suspension). Samples 1 and 3 were treated with non-radioactive iodoacetamide (2.5  $\mu\text{mol}$ ) for 20 min., washed once and then all samples were exposed to [ $^{14}\text{C}$ ]-iodoacetamide (2.66  $\mu\text{mol}$  at 0.94  $\mu\text{Ci}/\mu\text{mol}$  i.e.  $5.50 \times 10^6$  dpm/sample) for 1 hour before washing.

#### 4. Coupling of Papain to Bead-S-S-2-Py

The coupling of activated papain (36) to the present bead-S-S-2-Py preparation was carried out under conditions modelled after those of Brocklehurst and co-workers (26) with an agarose-glutathionyl-S-S-2-Py system. Papain activation, which involved treatment with low molecular weight thiol followed by gel filtration, yielded samples of the enzyme containing 0.43 and 0.52 moles of thiol per mole of protein of molecular weight 23,000. These values may be compared to literature ratios (36) in the range 0.4-0.6. The results of two coupling experiments are presented in Table VIII. Py-2-SH release in these experiments indicated 67 and 84% coupling whereas determination of the decrease in protein content suggested 41 and 55% coupling respectively. Bead supernatants after the DTE cleavage reaction could be checked for protein by measurement of  $A_{278}$ . When this was done the results of Expt. 1 showed that all of the protein coupled during Py-2-SH release was recovered upon DTE back reaction. By contrast the coupling supernatant of Expt. 2 showed only a small fraction of the expected protein. The uncertainty in both of these measurements lay in the fact that correction of  $A_{278}$  values due to Py-2-SH present was a large proportion of the absolute magnitude of those absorbances. Thus the cleavage supernatants were dialyzed exhaustively, until  $A_{343}$  values dropped to zero, and  $A_{278}$ 's were remeasured to give

Table VIII: Coupling of Papain with Bead-S-S-2-Py <sup>a</sup>

	Experiment 1	Experiment 2
Bead-S-S-2-Py	2.6 $\mu$ mol	3.0 $\mu$ mol
Sample Applied	Protein <sup>b</sup> 2.8 mg of which 1.5 mg (0.063 $\mu$ mol) was papain-SH	Protein <sup>b</sup> 2.9 mg of which 1.3 mg (0.055 $\mu$ mol) was Papain-SH
Coupling supernatant	0.042 $\mu$ mol Py-2-SH protein <sup>b</sup> 2.2 mg	0.046 $\mu$ mol Py-2-SH protein <sup>b</sup> 2.2 mg
DTE cleavage supernatant	protein <sup>b</sup> 1.0 mg(before dialysis) protein <sup>b</sup> 0.36 mg(after dialysis)	protein <sup>b</sup> 0.17 mg protein <sup>b</sup> 0.11 mg

a. Samples (6ml) of activated papain, taken directly from G-25 gel filtration in 0.05 M sodium acetate buffer of pH 4.5 containing 0.15 M NaCl and 1 mM EDTA were reacted with 2 ml packed bead-S-S-2-Py (40-50-fold excess of bead-S-S-2-Py) for 20 min. at room temperature. The supernatant was removed by filtration and the beads were washed with the same buffer. The two filtrates were pooled as "coupling supernatant". The beads were then treated with 15 mM DTE in 0.1 M sodium phosphate buffer of pH 7.55 for 30 min at room temperature. The supernatant was again removed by filtration and combined with a subsequent wash with the same solution and characterized as the DTE cleavage supernatant. This solution was dialyzed 2 days at 4°C against several changes of N<sub>2</sub>-saturated acetate

Table VIII: Coupling of Papain Bead-S-S-2-Py (cont'd)

buffer above.

- b. Protein was determined from  $A_{278}$  measurement. In cases where Py-2-SH was present  $A_{278, \text{corr.}}$  was calculated from  $A_{278, \text{corr.}} = A_{278} - 1.25 \times A_{343}$  (33)

the final values in Table VIII. The amounts of protein shown were 60 and 16% of the corresponding amounts of protein not present in the coupling supernatants and correspond to 28 and 7 nmol/g of dry beads, respectively. The same amounts were 37 and 10% of the degree of protein coupling indicated by Py-2-SH release.

#### 5. Coupling of BSA-SH to Bead-S-S-2-Py

Initial attempts to couple mercaptalbumin (BSA-SH) to bead-S-S-2-Py were formulated with conditions analogous to those of Carlsson and co-workers (22,23), for BSA-SH coupling to agarose-glutathionyl-S-S-2-Py beads. Thus coupling times of hours at room temperature and pH 7.5 with bead-S-S-2-Py groups in large (5-15-fold) excess were used. The BSA samples used in these experiments were characterized by a thiol titration of ca 0.4 mole thiol per mole of polypeptide of molecular weight 68,000. The results of three such experiments are given in Table IX. In each case it is seen that amounts of 2-thiopyridone were liberated which were nearly equal to or greater than the molar quantities of mercaptalbumin in the experiments and yet amounts of protein present in the coupling supernatants were not consistent with such large amounts of coupled protein. Examination of the DTE cleavage supernatants (after concentration and ultrafiltration to remove Py-2-SH) showed very small amounts of protein

Table IX: Coupling of BSA-SH with Bead-S-S-2-Py <sup>a</sup>

	Experiment 1	Experiment 2	Experiment 3
$\mu$ mol of bead-S-S-2-Py	0.49 $\mu$ mol	2.47 $\mu$ mol	3.15 $\mu$ mol
Sample applied	Protein 20 mg of which 6.8 mg (0.1 $\mu$ mol) was BSA-SH	Same	Same
Coupling supernatant	0.086 $\mu$ mol Py-2-SH protein <sup>b</sup> 15.5 mg	0.104 $\mu$ mol Py-2-SH protein <sup>b</sup> 16.7 mg	0.243 $\mu$ mol Py-2-SH protein <sup>b</sup> 17.3 mg
DTE cleavage supernatant	Protein <sup>b</sup> 0.105 mg	Protein <sup>b</sup> 0.408 mg	Protein <sup>b</sup> 0.3 mg

- a. All reactions were carried out at room temperature in 0.1 M sodium phosphate buffer of pH 7.55 containing 0.1 mM EDTA (a) 2.5 hr. for experiment 1 (17% suspension) (b) 16 hr. for experiments 2 and 3 (56% suspension). The coupling and DTE cleavage supernatants were removed as for the papain experiments (footnote to Table VIII).
- b. Protein was determined from  $A_{280}$  measurements on the supernatant solutions which had been concentrated and washed free of Py-2-SH in an ultrafiltration centrifuge cone apparatus.

bound. In the three experiments protein corresponding to 1.5, 6.0 and 4.4% of the available BSA-SH remained in the cleavage supernatant and these amounts corresponded to occupancy of 0.32, 0.24 and 0.14% of the available bead-S-S-2-Py sites, respectively. The amounts of protein in the cleavage supernatants corresponded to 9, 5 and 4 nmol/g of dry beads respectively.

Table X shows the results of coupling reactions formulated analogously to the foregoing but these were stopped after much shorter times (1-2 min. or 10 min.). One experiment was carried out at pH 7.5 while the other occurred in pH 4.5 buffer. The amounts of Py-2-SH in the coupling supernatants at 1-2 minutes already corresponded to binding of BSA-SH in greater than or equal to 1.0 mg amounts, i.e., greater than the amounts of protein isolated in the cleavage supernatants of the 3-16 hr reactions of Table IX. Coupling supernatants after 10 min indicated 2-thiopyridone concentrations more than 3-fold lower than those of the 1-2 minute reactions. The sum of the long and short period coupling reaction data pointed up the possibility that other complicating processes such as exchange reactions might be occurring in competition with the desired reaction. Further examination of BSA-SH and its derivatives in the following sections was aimed at determining whether the foregoing results reflected the bead reagents or the protein itself.

Table X: Coupling of BSA-SH with Bead-S-S-2-Py<sup>a</sup>

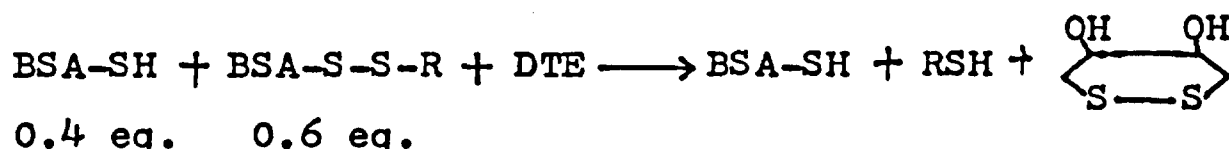
	Experiment 1	Experiment 2
$\mu$ mol of Bead-S-S-2-Py	2.3	4.3
Sample Applied	Protein 20 mg of which 6.8 mg (0.1 $\mu$ mol) was BSA-SH	Protein <sup>c</sup> 19.1 mg all of which (0.28 $\mu$ mol) was BSA-SH
Coupling Supernatant		
(a) 1-2 min Reaction time	0.035 $\mu$ mol Py-2-SH protein <sup>b</sup> 16.0 mg	0.013 $\mu$ mol Py-2-SH protein <sup>b</sup> 14.5 mg
(b) 10 min Reaction time	0.011 $\mu$ mol Py-2-SH protein <sup>b</sup> 16.8 mg	0.002 $\mu$ mol Py-2-SH protein <sup>b</sup> 13.6 mg

- a. All reactions were carried out at room temperature in (a) 0.1 M sodium phosphate buffer of pH 7.55 containing 0.1 mM EDTA and 0.3 M KCl for experiment 1 (40% suspension) (b) 0.05 M sodium acetate buffer of pH 4.5 containing 0.15 M NaCl for experiment 2 (36% suspension).
- b. Protein was determined from  $A_{278}$  measurement. In case where Py-2-SH was present  $A_{278}$ , corr. was calculated from  $A_{278}$ , corr. =  $A_{278} - 1.25 \times A_{343}$  (33).
- c. BSA was treated with 0.9 eq. of DTE and the solution removed by passage through a Sephadex G-25 Column. 0.05 sodium acetate buffer, pH 4.5, containing 0.15 M NaCl was used throughout. The thiol content of BSA-SH was 1.0 SH/68,000.



## 6. BSA-SH of High Thiol Titre and BSA-S-S-2-Py

BSA-SH used in most of the foregoing experiments (Except Expt. 2, Table X) carried 0.4 mol thiol per mole of protein of molecular weight 68,000. As evidence has been reported that the remaining protein in such samples has the sulfhydryl group masked as the mixed disulfide with small thiols such as cysteine and glutathione attempts were made to convert BSA as obtained to samples of high thiol titration by treatment with DTE as follows (23):



It was found that treatment with 0.6 eq. DTE increased the thiol titration to ca 0.8 eq./mol and that 0.9 eq. DTE was required to consistently yield protein samples showing greater than 0.9 eq./mol after gel filtration to remove small molecules. Such "activated" BSA-SH samples were added slowly to excess 2-Py-S-S-2-Py at pH 4.5 and then filtered on a Sephadex G-25 column to obtain samples characterized as BSA-S-S-2-Py on the basis of zero thiol titrations and release of Py-2-SH corresponding to 75-100% of the original thiol contents upon reduction with DTE. A qualitative indication of the rate of the BSA-S-S-2-Py formation reaction was gained by mixing the quantities all at once in a cuvette and

monitoring  $A_{343}$ . The increase in that absorbance was complete in less than 20 minutes at pH 4.5, room temperature.

#### 7. BSA-S-S-2-Py Coupling with Bead-SH

Excess Bead-SH was incubated at room temperature with BSA-S-S-2-Py samples as obtained above. Suspension pH was maintained at 4.5 and reaction period held to 10 minutes based on the qualitative indications of rates in the foregoing section. Results of three experiments are presented in Table XI. Examination of the coupling supernatants for the presence of Py-2-SH was not useful in these experiments because of the dilution of those solutions. For example,  $A_{343}$  of that supernatant for Expt. 1, where the largest coupling was observed was 0.026 (1 cm pathlength). There it may be seen that, despite 18, 4.3 and 5.4-fold respective excesses of bead thiol over protein-S-S-2-Py groups, reasonable amounts of protein were coupled in at least two of the three cases. Calculation indicates that respectively 15, 1.3 and 3.9% of the protein available as BSA-S-S-2-Py was detected in the cleavage supernatant, that these amounts corresponded to occupancy of 0.8, 0.3 and 0.7% of the bead-SH sites, respectively, and that 59, 15 and 36 nmol of BSA per gram of dry beads were coupled, respectively.

Table XI: BSA-S-S-2-Py Coupling with Bead-SH <sup>a</sup>

	Experiment 1	Experiment 2	Experiment 3
Bead-SH	1.20 $\mu$ mol	0.79 $\mu$ mol	0.80 $\mu$ mol
Sample applied	protein 8.1 mg of which <del>4.5 mg</del> (0.066 $\mu$ mol) was Protein-S-S-2-Py	Protein 14.8 mg of which 12.5 mg (0.184 $\mu$ mol) was Protein-S-S-2-Py	Protein 14.5 mg of which 10.0 mg (0.147 $\mu$ mol) was Protein-S-S-2-Py
Coupling supernatant	0.019 $\mu$ mol Py-2-SH Protein <sup>b</sup> 6.9 mg	0 $\mu$ mol Py-2-SH Protein <sup>b</sup> 10.4 mg	0 $\mu$ mol Py-2-SH Protein <sup>b</sup> 12.0 mg
DTE cleavage supernatant	Protein <sup>b</sup> 0.68 mg	Protein <sup>b</sup> 0.16 mg	Protein <sup>b</sup> 0.39 mg

- a. All reactions were carried out at room temperature in 0.05 M sodium acetate of pH 4.5 containing 0.15 M NaCl for 10 min.
- b. Protein was determined from  $A_{278}$  measurement. In cases where Py-2-SH was present  $A_{278}$ , corr. was calculated from  $A_{278}$ , corr. =  $A_{278} - 1.25 \times A_{343}$  (33).

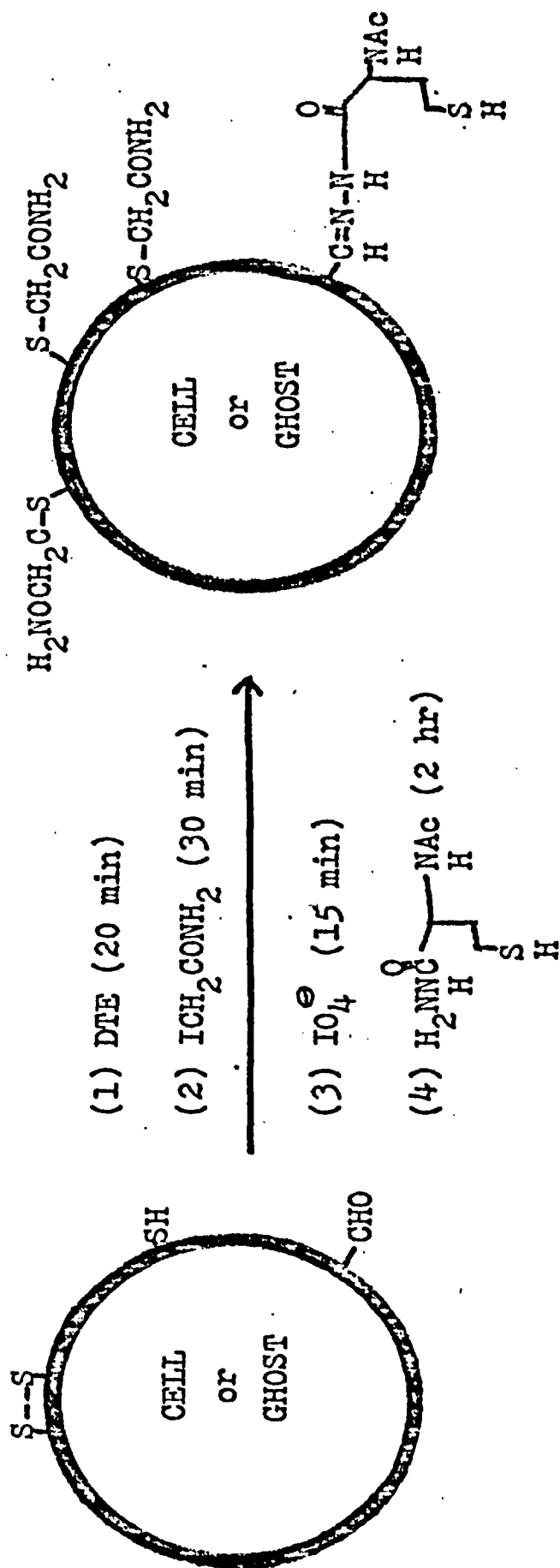
## 8. BSA-S-S-2-Py Reaction with BSA-SH

BSA-SH (0.1 ml, 0.027  $\mu$ mol SH) and BSA-S-S-2-Py (1 ml, 0.027  $\mu$ mol -S-S-2-Py) (in 0.05 M sodium acetate buffer containing 0.15 M NaCl and 1 mM EDTA, pH 4.5, at room temperature) were mixed immediately in a cuvette and followed at 343 nm. Py-2-SH release leveled off at about  $16.5 \pm 2\%$  (duplicate reactions), within 20 minutes and remained constant for at least 40 min. After gel filtration on Sephadex G-25 the samples were checked for the presence of increased numbers of dimers by electrophoresis on polyacrylamide in the presence of SDS and absence of reducing agent. In all cases the amount of dimer present was identical (as determined by electrophoresis) with that of a BSA sample subjected directly to G-25 filtration.

## 9. Cell Coupling

The sulfhydryl group content of ghosts was determined by DTNB and 2-Py-S-S-2-Py titration with and without SDS present. The results were  $0.28 \pm 0.01 \mu$ mol/mg, and  $0.14 \pm 0.01 \mu$ mol/mg of membrane protein respectively.

A plan for blocking endogeneous membrane thiol and introducing thiol into the carbohydrate of glycoproteins using a thiol hydrazide reagent developed by Y.C. Wu in this laboratory is shown in Scheme XI. After the ghost sample was treated with iodoacetamide, the sulfhydryl group titre



Scheme XI: Reaction of Ghost with Thiol Hydrazide (All Operations at Room Temperature and pH 7.5)

was lowered to  $0.063\mu\text{mol/mg}$  of membrane protein. Subsequent treatment with thiol hydrazide raised the sulfhydryl group titre to  $0.313\mu\text{mol/mg}$ . The latter two determinations were performed in the absence of SDS.

Ghost-hydrazone (4.8 mg,  $1.5\mu\text{mol}$  ghost-SH) was incubated with bead-S-S-2-Py ( $3.15\mu\text{mol}$ ) for 30 min at room temperature, pH 7.5. Py-2-SH release in the coupling supernatant was  $33.0\text{ nmol}/0.7\text{ g}$  of dry beads. After extensive washing in the absence and then presence of SDS the bead sample was subjected to a DTE cleavage step. The amount of protein found in the supernatant was 0.29 mg which was 6.1% of the amount of protein in this experiment.\*

---

\* In conjunction with other work, a sample of the DTE cleavage supernatant was subjected to SDS-poly-acrylamide gel electrophoresis by K. E. Taylor using the Laemmli system (47). Virtually the only Coomassie blue-staining material on the gel barely entered the separating gel and thus could be judged to have a molecular weight well in excess of the erythrocyte bands 1 and 2 (41) which have been estimated at 220,000 and 250,000 Daltons.

## CHAPTER IV

### DISCUSSION

#### A. Preparation of Long-Arm Thiolated Beads

Of the steps comprising the general strategy of Scheme V, the alkylation reaction was investigated first as it was expected that the preceding acylation reaction would be relatively insensitive to the halogen atom present. In the alkylation study, Table I, displacement of bromide or iodide were found to be much faster than chloride even when the latter occurred in the presence of potassium iodide. Use of KI as a catalyst in alkylations on sulfur was reported by Schechter et al.(35), but their experiments were on a different time scale(several days) and involved alkylation of a sulfide. It is also clear from Table I that alkylation by iodoacetamide, which is a better model for the intended bead system, is much faster than the corresponding reaction with iodoacetic acid.

Chloroacetylation of aminoethyl beads via three commonly used peptide coupling reagents was surveyed, Table II, in each case with a 10-20-fold excess of acyl source over bead amino groups. With the water-soluble carbodiimide, EDAC, coupling yield was lower(35%) than with the reagent K or NHS-ester methods(60,66%). No further effort was directed to the use of EDAC as reagent stability and purity as supplied were variable. Reagent K was not further employed due to cost of

the reagent. Haloacetylation via the NHS-ester showed no dramatic dependence on the halogen atom present.

The foregoing comments indicate that in all cases significant fractions of the original amino group titres were not acylated. In order that these not act as possible sites for electrostatic interactions with macromolecules, haloacetyl beads were routinely treated with acetic anhydride. This reagent when used in formamide, a solvent suggested by the manufacturer not to cause shrinkage of the beads, resulted in masking around 90% of the remaining bead amino groups.

Reaction of the three types of haloacetamido beads with the long arm  $\alpha,\omega$ -dithiol, Table III, showed little difference in the coupling yields from the bromo- and iodo-derivatives where levels of 12-14  $\mu\text{mol/g}$  were achieved thus representing 12-15% of the sites available. Surprisingly, in the light of the alkylation study (Table I), the chloroacetamido beads in the presence of KI coupled lesser but still reasonable quantities of dithiol. Nevertheless, it was decided not to continue use of the chloroacetamido beads.

An indication of the accessibility of bead haloacetamido groups was obtained by comparison of the results of a small molecule, dithioerythritol, with results for the long arm dithiol discussed above (Scheme IX and Table IV). In three preparations varying in levels of initial activation the smaller dithiol reached 2-fold higher coupling yields.



This greater accessibility of bead haloacetyl groups to small thiol was the result which provoked introduction of an additional blocking reaction: beads were subjected to high mercaptoethanol concentration after long-arm dithiol coupling, in order to discharge as many unreacted haloacetyl groups as possible.

The use of bromo- vs iodo-acetamido groups in the bead elaboration was not made on the results shown in Tables III and IV where little distinction may be made. Rather, the iodo-derivative was chosen over the bromo solely because bromoacetic acid is a waxy, sticky solid which is less easily dispensed than iodoacetic acid.

In summary, the tactics used to best effect the strategy outlined in Scheme V are depicted in Scheme X and three preparations which were processed directly through that set of steps are represented in Table V. There, and in the last preparation of Table IV it may be noted that initial levels of bead aminoethylation used were lower, 30-50  $\mu\text{eq/g}$ , than in previous preparations, 78-184  $\mu\text{eq/g}$ . Lower levels of activation were chosen upon the realizations: (a) that in all cases less than 15% of the available bead haloacetamido groups were accessible to a dithiol of average molecular weight around 700 Daltons, and (b) calculations summarized in the following paragraph indicated functional group densities on "hard-sphere" beads which would be saturating around the

levels shown in Table V.

In Appendix 2a, a calculation has been carried out based on non-porous beads of  $25 \times 10^{-6}$  m diameter, assuming 74% space occupancy for close packing, and using the manufacturer's water regain data to estimate the number of beads per dry gram. There it is shown that for surface functional group densities of 2 and 12  $\mu\text{eq/g}$  "patches" of surface  $7.2 \times 7.2 \text{ \AA}$  and  $2.9 \times 2.9 \text{ \AA}$  respectively would be available per functional group. Two recent literature reports are relevant to these calculations. Jacobson and Branton(45), using the same type of beads estimated a bead surface area for  $30 \mu\text{m}$  diameter beads which was about 40% lower than could be estimated by the above type of calculation. Whitesides and co-workers(46) found saturation of polyethylene surface functional groups(carboxyl groups in their study) at densities of one group per  $4 \text{ \AA}^2$ . Since this study is focused on introduction of bead surface functional groups for the purpose of attachment to globular proteins, either membrane-associated or free in solution, it is probable that even functional group densities of 2  $\mu\text{eq/g}$  are excessive with the beads presently used. For example, the second part of Appendix 2 shows a calculation for a spherical protein  $50 \text{ \AA}$  in diameter which indicates that if the surface of a  $25 \mu\text{m}$  diameter bead were completely hidden by protein molecules only 2.6% of the sites on beads of 2  $\mu\text{eq/g}$  group density would be utilized, assuming a single covalent attachment per protein molecule.

## B. Thiol-Disulfide Interchange and Alkylation Reactions of Long-Arm Thiolated Beads

Use of the beads discussed in the foregoing sections was mainly in the mixed activated disulfide form, bead-S-S-Ar, where in most cases the aryl group was the 2-pyridyl group. Accessibility of the bead-S-S-Ar to exchange with "small" thiols was probed in two ways: (a) free long-arm dithiol, PEG-DMP, in thiol:disulfide ratios from 1.7 to 44 in all cases showed release of  $91 \pm 3\%$  of the theoretical 2-thiopyridone. Thus, all bead-S-S-Ar are accessible; (b) mercaptoethanol in the presence of 20-fold excess of bead-S-S-Ar resulted in expulsion of 0.7 equivalents of 2-thiopyridone in minutes under the same conditions. Thus the interchange reaction itself is rapid.

As an independent probe of bead-SH reactivity and as a check on the spectrophotometric methods used for thiol determination on bead samples radiochemical titration was carried out using excess  $[1-^{14}\text{C}]$ -iodoacetamide of known specific activity. These experiments, Table VII, showed that incorporation required the presence of free thiol and reached levels amounting to 40-43% of those determined by 2-Py-S-S-2-Py titration. The discrepancy in these two types of thiol titration may reflect accessibility differences of the two reagents or, more likely, a degree of non-specific adsorption of 2-Py-S-S-2-Py to the polyacrylamide beads. Such adsorbed

aryl disulfide, if not subsequently washed away, would result in elevated Py-2-SH release during DTE back titration..

Accessibility of bead-S-S-2-Py groups was next probed using the soluble enzyme papain, a single chain globular protein of molecular weight 23,000 Daltons which contains a single sulfhydryl group somewhat buried in the active site. In experiments, Table VIII, modelled after those used for the agarose-based gels(26), with bead-S-S-2-Py in large excess(40-50-fold) over protein thiol, thiopyridone release corresponding to 67 and 84% of protein thiol groups occurred in minutes at pH 4.5. By contrast, examination of the amount of protein remaining in the coupling supernatants indicated that the above coupling yields were overestimates by a factor of 1.5. Furthermore, estimation of protein in the DTE cleavage supernatants showed further reduction of 1.6- and 6-fold, respectively, in the amounts of protein actually isolated. It may be noted that coupling yields indicated by thiopyridone release, approximately 40-50 nmol/g of dry beads, are approaching the above-calculated saturation of the bead surface with globular proteins(assuming hard-sphere geometries). Coupling yields based on protein present in the DTE cleavage supernatant were 28 and 7 nmol/g. For comparison, capacity of the macroporous agarose-glutathionyl-S-S-2-Py beads for papain was 10% of theoretical or 200-400 nmol/g(19). The discrepancies in coupling yields indicated by thiopyridone release vs the actual amounts of protein isolated amount to

2.6- and 9.6-fold in the experiments of Table VIII. However, it is suggested that these are not large given the scale of the experiments and that additional refinement of the workup procedure may lead to markedly better recoveries.

Attention was next turned to another soluble protein, bovine serum albumin, whose size is closer to that of many membrane proteins. This single polypeptide of 68,000 Daltons molecular weight contains a single sulfhydryl group and seventeen disulfide pairs. However, it is only available in a form in which ca. 60% of the single sulfhydryl groups are blocked as mixed disulfides with cysteine or glutathione. Polypeptides containing a complete equivalent of sulfhydryl per 68,000 Daltons, referred to as mercaptalbumin, have been isolated from partially blocked preparations via covalent chromatography on glutathionyl-agarose(22,23) as described in the INTRODUCTION. Thus initial experiments with the present mixed activated disulfide beads, Table IX, were patterned after those successful with the agarose-based system. Thiopyridone release up to 2-fold higher than the amount of mercaptalbumin in the experiment and 2-4-fold higher than the approximate saturation limit for bead surface coverage was the first indication of complicating factors. The amounts of protein in the cleavage supernatants, by contrast, were 20- to 55-fold lower than expected from thiopyridone release and 6- to 12-fold lower than the approximate saturation limit. Additional experiments were carried out at pH's 7.55 and 4.5

but for much shorter times, Table X, in an attempt to survey conditions successful in the papain coupling experiment. Sizeable thiopyridone release was observed within 2 minutes which in the ensuing 8 minutes decreased 3-6-fold and this phenomenon was observed whether or not the BSA sample was pre-treated with DTE before attempted coupling (Table X, Expt. 2 vs Expt. 1).

When the mode of coupling was reversed, that is, bead-SH coupling with protein-S-S-2-Py instead of bead-S-S-2-Py with protein-SH as hitherto discussed, the results with BSA were much more encouraging (Table XI), showing amounts of protein in the DTE cleavage supernatant amounting to greater than 30 nmol of BSA per gram of dry beads in two of the cases.

The contrasting results of BSA coupling to beads in the two different modes could possibly reflect properties of BSA itself. One further experiment, that of attempting to couple BSA-SH with BSA-S-S-2-Py to form dimers, was carried out to explore this possibility. There, pyridone release was not accompanied by any detectable (by gel electrophoresis) increase in the dimer content of BSA. Norris & Brocklehurst (24) have shown that the reactivity of the BSA thiol group is extremely high with a second-order rate constant approaching that of cysteine and some 10-fold higher than that of the papain active site sulfhydryl. Thus, the failure of the BSA-SH plus bead-S-S-2-Py coupling experiments

might be due to the extreme accessibility of that sulfhydryl on the native polypeptide which allows a manifold of subsequent thiol-disulfide interchanges.

Bead-cell coupling based on a reaction between two solids was attempt via directed disulfide formation in the present work. The sulfhydryl group content of ghosts was 2-fold higher in the presence than in the absence of SDS. Bead-cell coupling was performed in the absence of SDS, but under these conditions it is not clear what proportion of the titratable sulfhydryl groups would be accessible to bead reagents approaching only from the outside. For this reason the ghosts were first reduced and alkylated in order to mask a large proportion of the endogenous thiols and disulfides. Then, after mild periodate oxidation, thiol was introduced, presumably via cell surface oligosacchorides, using a hydrazone-forming reagent developed in this lab. This treatment did not simply increase the membrane thiol content 5-fold but did so in a way which should leave the mercapto groups accessible to large reagents. Py-2-SH release into the coupling supernatant was 47 nmol per gram dry beads which amounts to 7 nmol per mg of membrane protein. The amount of protein found in the supernatant corresponded to 6.1% of the amount of protein in this experiment.

It is interesting to note that thiopyridone release during coupling also approached the saturation limit calculated

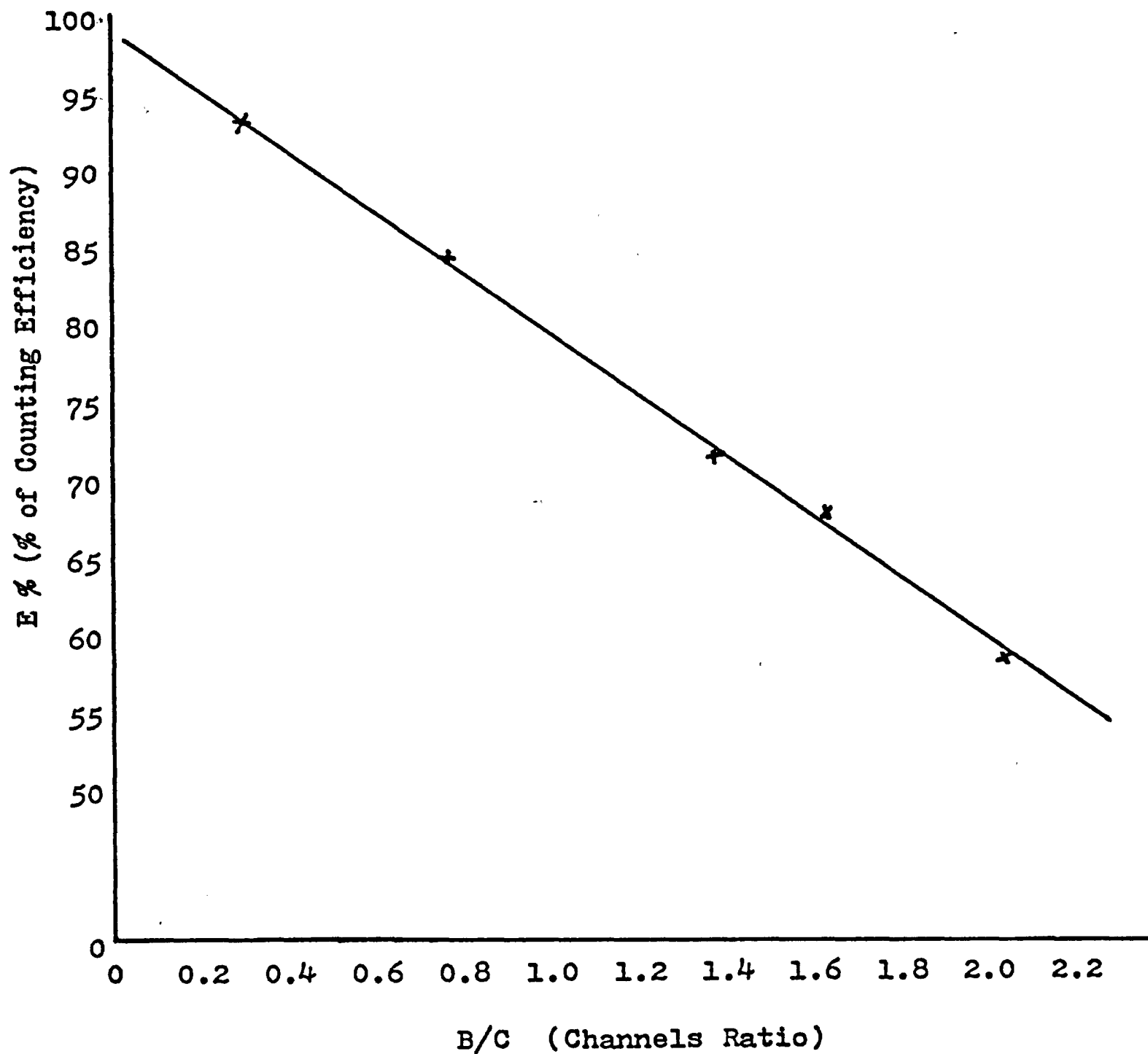
on a hard sphere model (Appendix 2b) even though those calculations were carried out for packing of a soluble, globular protein around the bead surface. It might be speculated that a similar degree of cell accessibility reflects interaction of functional groups on complementary long spacer arms--the thiolated oligosaccharides on the cells and activated mixed disulfides on the bead poly(oxyethylene) spacers.

The bead-cell coupling results, although preliminary, are encouraging enough to warrant further evaluation of their use in isolation of membrane glycoproteins.



## APPENDIX 1

Quench Correction Curve Using A Set of  
 $^{14}\text{C}$  Quenched Standards from Nuclear Chicago



## APPENDIX 2

### (a) Calculation of Functional Group Density on Bead Surface

(i) For spherical beads of  $25 \times 10^{-6}$  m diameter:

$$\text{Area per bead} = 4\pi r^2 = 1.96 \times 10^{-9} \text{ m}^2 = 1.96 \times 10^{+11} \text{ \AA}^2$$

$$\text{Volume per bead} = 4\pi r^3/3 = 8.18 \times 10^{-15} \text{ m}^3$$

1 gram of dry beads results in 3.5 ml of packed, wet beads (from Bio Rad water regain data for Biogel P-2) and of this volume 74% or  $2.59 \times 10^{-6} \text{ m}^3$  would be due to beads themselves, assuming close packing of spheres.

$$\text{Beads per dry gram} = \frac{2.59 \times 10^{-6}}{8.18 \times 10^{-15}} = 3.17 \times 10^8$$

$$\begin{aligned} \text{Total surface area per dry gram} &= 3.17 \times 10^8 \times 1.96 \times 10^{-9} \text{ m}^2 \\ &= 6.22 \times 10^{-1} \text{ m}^2 \\ &= 6.22 \times 10^{19} \text{ \AA}^2 \end{aligned}$$

2  $\mu$ eq functional groups per dry gram correspond to  $1.2 \times 10^{18}$  sites per gram and if these sites are on the bead surface this corresponds to 51.8  $\text{\AA}^2$  per site or a "patch" of surface  $7.2 \times 7.2 \text{ \AA}$ .

12  $\mu$ eq functional groups per dry gram would similarly correspond to 8.63  $\text{\AA}^2$  per site or a "patch" 2.9x2.9  $\text{\AA}$ .

(ii) For spherical beads of  $10 \times 10^{-6}$  m diameter:

2  $\mu$ eq/g corresponds to 130  $\text{\AA}^2$  of surface per site or a "patch" 11.4x11.4  $\text{\AA}$ .

(iii) For spherical beads of  $2 \times 10^{-6}$  m diameter:

2  $\mu$ eq/g corresponds to 648  $\text{\AA}^2$  of surface per site or a "patch" 25.4x25.4  $\text{\AA}$ .

#### (b) Calculation of Packing of Spherical Protein Molecules on Surfaces of Spherical Beads

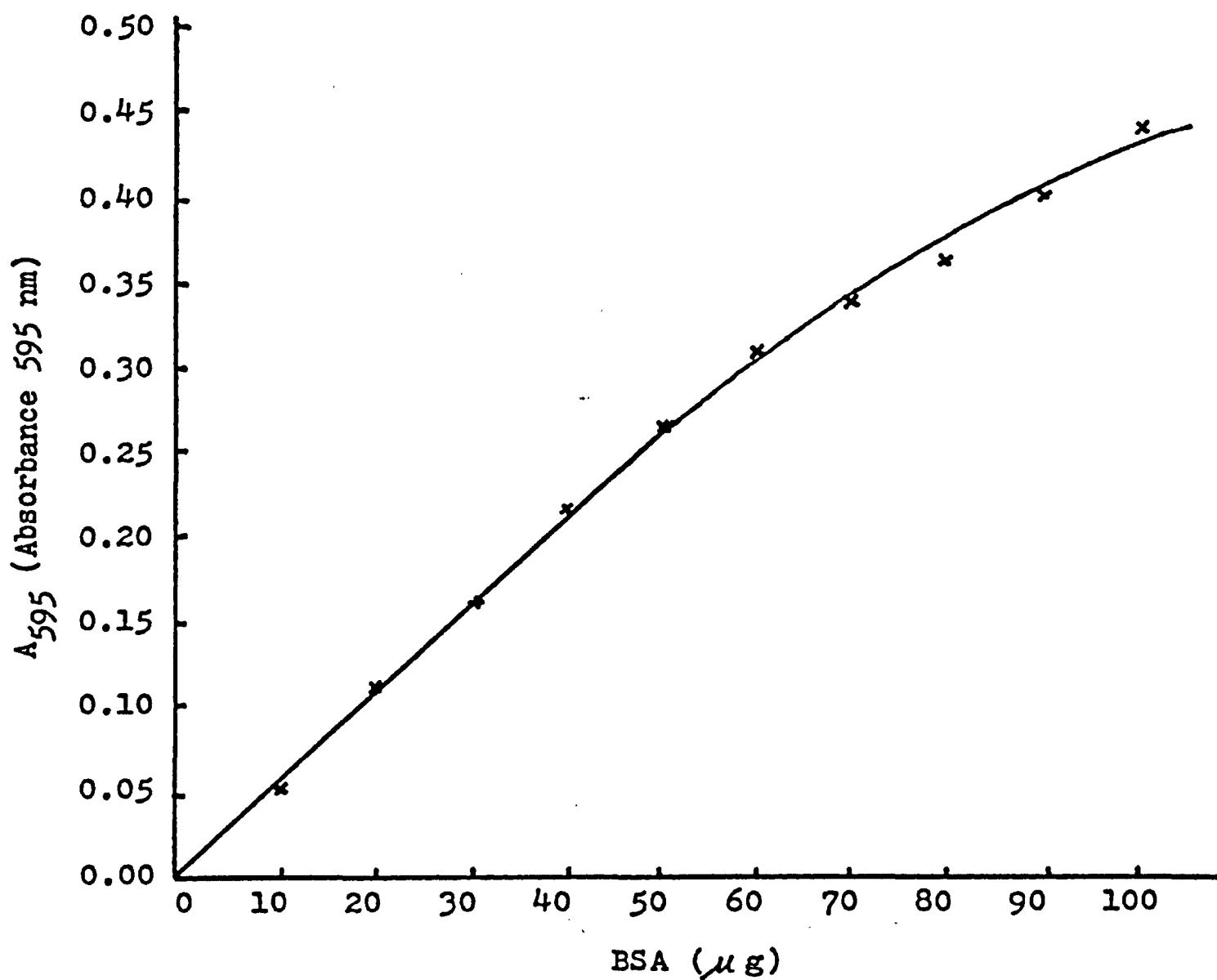
A spherical protein of 50  $\text{\AA}$  diameter (40-50,000 Daltons) would have a cross-sectional area of 1964  $\text{\AA}^2$  and if this area is taken as the "projection" of such a globular protein packed around a bead:

(i) For spherical beads of  $25 \times 10^{-6}$  m diameter (see (a), (i), above) the maximum amount of protein per gram of dry beads would 53 nmol, which, for beads with 2  $\mu$ eq/g of surface functional groups, represents 2.7% utilization of bead sites at saturation, for mono-attachment of each protein molecule.

(ii) For spherical beads of  $2 \times 10^{-6}$  m diameter with 2  $\mu$ eq/g of surface functional groups the analogous calculation indicates 33% utilization of bead sites at saturation.

### APPENDIX 3

BSA Standard Curve Using Coomassie Brilliant  
Blue G-250 of Bradford's Method (40)



## REFERENCES

1. Campbell D.H., Leuscher E., and Lermann L.S., Proc. Nat. Acad. Sci., U.S.A., 37, 575 (1951).
2. Sander E.G., McCormick D.B., and Wright L.D., J. Chromatogr., 21, 419 (1966).
3. Erhan S.L., Northrup L.G., and Leach F.R., Proc. Nat. Acad. Sci., U.S.A., 53, 646 (1965).
4. Lerman L.S., Proc. Nat. Acad. Sci., U.S.A., 39, 232 (1953).
5. Arsenis C., and McCormick D.B., J. Biol. Chem., 239, 3039 (1964); 241, 330 (1966).
6. Cuatrecasas P., Wilchek M., and Anfinsen C.B., Proc. Nat. Acad. Sci., U.S.A., 61, 636 (1968).
7. Cuatrecasas P., and Wilchek M., Biochem. Biophys. Res. Commun., 33, 235 (1968).
8. Chan W.C., and Takahashi M., Biochem. Biophys. Res. Commun., 37, 272 (1969).
9. Cuatrecasas P., J. Biol. Chem., 245, 3059 (1970).
10. Cuatrecasas P., Advances in Enzymology, 36, 29 (1972).

11. Cuatrecasas P., and Anfinsen C.B., Ann. Rev. Biochem., 40, 259 (1971).
12. Madelian V., and Warren W.A., Anal. Biochem., 64, 517 (1975).
13. Frost R.G., Reitherman R.W., Miller A.L., and O'Brien J.S., Anal. Biochem., 69, 170 (1975).
14. Guyda H.J., and Friesen H.G., Biochem. Biophys. Res. Commun., 41, 1068 (1971).
15. Edelman M., Anal. Biochem., 65, 293 (1975).
16. Cuatrecasas P., and Anfinsen C.B., Methods in Enzymology, 22, 345 (1971).
17. Inman J.K., and Dintzis H.M., Biochemistry, 8, 4074 (1969).
18. Inman J.K., Methods in Enzymology, 34, 30 (1974).
19. Brocklehurst K., Carlsson J., Kierstan P.J., and Crook E.M., Biochem. J., 133, 573 (1973).
20. Brocklehurst K., Carlsson J., Kierstan P.J., and Crook E.M., Methods in Enzymology, 34, 531 (1974).
21. Brocklehurst K., and Little G., Biochem. J., 133, 67 (1973).

22. Carlsson J., and Svenson A., FEBS LETTERS, 42, 183 (1974).
23. Egorov T., Svenson A., Ryden L., and Carlsson J., Proc. Nat. Acad. Sci., 72, 3029 (1975).
24. Norris R., and Brocklehurst K., Biochem. J., 159, 245 (1976).
25. Malthouse J., and Brocklehurst K., Biochem. J., 159, 221 (1976).
26. Shipton M., and Brocklehurst K., Biochem. J., 171, 385 (1978).
27. Carlsson J., Drevin H., and Axen R., Biochem. J., 173, 723 (1978).
28. (a) Separation News (2) (1979) Pharmacia Fine Chemicals AB.
- (b) Ghetie V., Mota G., and Sjöquist J., J. Immunol. Methods, 21, 133 (1978).
- (c) Dvorak D.J., Gipps E., and Kidson C., Nature, 271, 564 (1978).
- (d) Eshhar Z., Waks T., and Bustin M., Meth. Enzymol., Vol. XXXIV. Jakoby W.B., and Wilchek M. (Eds), AP, New York, 750 (1974).
29. Kahlenberg A., Anal. Biochem., 74, 337 (1976).

30. Shami Y., Ship S., and Rothstein A., Anal. Biochem., 80, 438 (1977).
31. Wu Y.C., M.Sc., University of Windsor, 1979.
32. Ellman G.L., Arch. Biochem. Biophys., 82, 70 (1959).
33. Grassetti D.R., and Murray J.F., Arch. Biochem. Biophys., 119, 41 (1967).
34. Stuchbury T., Shipton M., Norris R., Malthouse J.P.G., Brocklehurst K., Herbert J.A.L., and Suschitzky H., Biochem. J., 151, 417 (1975).
35. Schechter Y., Rubinstein M., and Patchoinik A., Biochemistry, 16, 1424 (1977).
36. Brocklehurst K., and Little G., FEBS LETTERS, 9, 113 (1970).
37. Dreuth J., Jansonius J.N., Koekoek R., Swen H.M., and Wolters B.G., Nature, 218, 929 (1968).
38. Mitchel R.E.J., Chaiden I.M., and Smith E.L., J. Biol. Chem., 245, 3485 (1970).
39. Kirschenbaum D.M., (1970) in Handbook of Biochemistry, Selected Data for Molecular Biology (Sober, H., ed.), 2nd ed., pp. C-71-C-98, Chemical Rubber Company, Cleveland, Ohio.



40. Bradford M., Anal. Biochem., 72, 248 (1976).
41. Fairbanks G., Steck T.L., and Wallach D.F.H.,  
Biochemistry, 10, 2606 (1971).
42. Weber K., and Osborn M., J. Biol. Chem., 244, 4406 (1969).
43. Timkovich R., Anal. Biochem., 79, 135 (1977).
44. Li C.H., Group B., Chung D., and Ramachandran J.,  
J. Org., 28, 178 (1963).
45. Jacobson B.S., and Branton D., Science, 195, 302 (1977).
46. Rasmussen J.R., Stedronski E.R., and Whitesides G.M.,  
J. Amer. Chem. Soc., 99, 4736 (1977).
47. Laemmli U.K., and Favre M., J. Mol. Biol., 80, 575 (1973).

## VITA AUCTORIS

Name: Linda Mei Chien Chen

Date of Birth: July 10, 1949--Kwangtung, China. Daughter  
of Liang Hao Tong and Chi Yuin (Mao) Tong

Marital Status: Married, May 1975

Education: Bachelor of Science (Chemistry), Providence  
College, Taichung, Taiwan, R.O.C., June 1971

Awards: University of Windsor Scholarship, 1976-1978

Working Experience: Chinese Petroleum Corporation, Northern  
Engineering and Construction Div.,  
Taipei, Taiwan, R.O.C., Chemist,  
July 1971-Nov. 1974.